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ROLE OF AUTOPHAGY IN CANCER

by

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## ABSTRACT OF THE DISSERTATION

### Role of Autophagy in Cancer

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(Macro)autophagy is a catabolic process whereby intracellular components are enclosed into autophagosomes and delivered to lysosomes for degradation. Constitutive activity of autophagy contributes to turnover of proteins and organelles, ensuring the quality control of cellular components. Autophagy also can be induced by starvation or other stresses. This activity serves to provide internal resources to sustain metabolism and to prevent accumulation of detrimental substances. Therefore, autophagy is critical for cells to maintain homeostasis and to survive stress.

Tumors are often subjected to metabolic stress due to insufficient vascularization. Autophagy is induced and localizes to these hypoxic regions where it supports survival. In aggressive tumors, the increased metabolic demand of rapid proliferation and growth may augment the dependency of cells on autophagy. In addition, autophagy that is induced by cancer therapy may be utilized by tumor cells for survival and be counterproductive to therapeutic efficacy. In this work, we tested the hypothesis that autophagy enables tumor cell survival and tumorigenesis in two different settings and addressed the underlying mechanism by which this occurs.

First, we demonstrated that autophagy is required for viability in starvation and tumorigenicity of cells with oncogenic Ras activation. In these cells, defective autophagy caused abnormal mitochondria accumulation and reduced mitochondrial functionality in starvation associated with reduced energy charge. Since mitochondrial function is required for survival during starvation, we reasoned that autophagy supports survival and tumorigenicity of Ras-expressing cells by maintaining mitochondrial functionality. We also demonstrated that autophagy maintained mitochondrial function by preserving functional mitochondrial pools through mitophagy as well as by providing substrates for mitochondrial bioenergetic production under stress, thereby identifying autophagy and mitophagy as potential targets for treatment of cancers with oncogenic Ras activation.

Second, we examined the significance of the mTOR inhibition-induced autophagy in counteracting the efficacy of the mTOR inhibitor CCI-779, which has shown temporary effectiveness in clinical treatment of human renal cell carcinoma. We demonstrated that mTOR inhibition promoted autophagy-mediated stress tolerance. Inhibition of autophagy with chloroquine enhanced the cytotoxicity of CCI-779 in vitro and in allograft tumors in mice. We further demonstrated that autophagy promoted cell survival in CCI-779-treated cells by providing alternative substrates for mitochondrial metabolism and suppressing reactive oxygen species production. This result justified a combination of autophagy inhibition with mTOR inhibitors in cancer therapy.



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# **ROLE OF AUTOPHAGY IN CANCER**

## **Chapter 1. Introduction**

### **Overview**

Autophagy is a catabolic process by which cells degrade intracellular components in lysosomes. It includes three different variants: macroautophagy, microautophagy, and chaperone-mediated autophagy. Among them, macroautophagy (hereafter referred to as autophagy) is the most extensively studied form, which engulfs intracellular constituents into double membrane vesicles, termed autophagosomes, which then fuse with lysosomes to form autolysosomes, where the autophagic contents are degraded. Distinct from proteasomal degradation, which only proteolyze soluble proteins inside the proteasomal barrels, autophagy is the only cellular mechanism for degrading large cellular components such as protein aggregates and entire organelles. The substrates of autophagy may include cytoplasm, organelles, proteins and protein aggregates as well as the autophagic components that associated with the inner membranes of autophagosomes (Figure 1.1).

The cellular garbage disposal and intracellular recycling provided by autophagy serves to maintain cellular homeostasis by eliminating superfluous or damaged proteins and organelles, and invading microbes, or to provide substrates for energy generation or biosynthesis in stress. Thus, autophagy promotes the health of cells and animals and is critical for development, differentiation, maintenance of cell integrity and function as well as for the host defense against pathogens. Deregulation of autophagy is linked to susceptibility to various disorders including degenerative diseases, metabolic syndrome,

aging, infectious diseases and cancer. Given that cancer is a complex process and autophagy exerts its effects in multiple ways, role of autophagy in tumorigenesis is context-dependent. Our understanding of the molecular mechanism and regulatory pathways of autophagy has substantially improved by studies of numerous investigators over the past thirty years. The next challenge will be to understand the physiological significance of autophagy in various cellular contexts as well as how to modulate autophagy for therapeutic purposes in these settings.

**Figure 1.1. Molecular machinery and small molecule modulators of autophagy.**

The events of autophagosome formation- nucleation, elongation and maturation are depicted along with molecular machinery that regulates this process. The major negative regulator of autophagy, mTORC1, which integrates stimuli including availability of nutrients or growth factors, energy depletion and hypoxia, is also shown. Green or Red boxes indicate autophagy stimulators or inhibitors respectively. Question marks highlight potential therapeutic targets, the kinase Ulk1, the cysteine protease Atg4 and the E1-like ubiquitination enzyme Atg7, for which no drugs presently exist. PE: phosphoethanoamine. IP<sub>3</sub>: inositol-1,4,5-triphosphate. IP<sub>3</sub>R: inositol-1,4,5-triphosphate receptor. CBZ: carbamazepine. HCQ: hydroxychloroquine.



## **Molecular machinery of autophagy**

The central machinery of autophagy includes a series of complexes comprised of autophagy-related (Atg) proteins, executing the formation of autophagosomes: membrane nucleation, expansion, closure and maturation by fusing with lysosomes (Figure 1.1). In mammals, the Atg1/Ulk1 (Unc51-like kinase 1; the mammalian homolog of yeast Atg1) complex consisting of a kinase Ulk1, two scaffold proteins Atg13 and FIP200 (focal adhesion kinase family interacting protein of 200-kDa) and a recent identified component Atg101 receives signals from mTORC1 (mammalian target of rapamycin complex 1), an integrator of growth factors, nutrients and energy status (Mercer et al., 2009; Yang and Klionsky, 2010). During nutrient replete conditions, mTORC1 phosphorylates Ulk1 and Atg13 and negatively regulates Ulk1 complex. Upon starvation, dissociation of mTORC1 followed by a series of dephosphorylation/phosphorylation events activates the Ulk1 complex (Hosokawa et al., 2009; Jung et al., 2009). Although the direct targets of Ulk1 complex have not been identified, current evidence supports its roles in activation of Beclin1/Vps34 (Bcl-2 interacting coiled-coil protein 1/ vacuolar protein sorting 34) complex and thus contributes to autophagosome induction as well as maturation (Neufeld, 2010).

Several Beclin1-containing complexes that dictate the sequential process of autophagosome formation have been identified. The core components include the membrane anchor p150, Beclin1, and the class III phosphatidylinositol-3 kinase (PI3KC3) Vps34, which generates phosphatidylinositol 3-phosphate for protein recruitment. In addition, Atg14L (Atg14-like; the mammalian homolog of yeast Atg14) and UVRAG (UV irradiation resistance-associated gene) interact with Beclin1 in a

mutually exclusive fashion. The Beclin1/Vps34/Atg14L complex is involved in autophagosome initiation. UVRAG binds Bif-1 (Bax-interacting factor 1; endophilin B1), which deforms membrane and is involved in membrane elongation. The UVRAG complex also promotes the fusion of autophagosomes with lysosomes. As a negative regulator, Rubicon (RUN domain and cysteine-rich domain containing, Beclin1-interacting) inhibits the function of UVRAG complex in autophagosome maturation (Matsunaga et al., 2009; Zhong et al., 2009). In nervous system development, another Beclin1 interacting protein, Ambra1 (activating molecule in Beclin1-regulated autophagy) stimulates autophagy through promoting the interaction between Beclin1 and Vps34 and provides a possible tissue specific regulation of autophagy (Cecconi and Levine, 2008).

Two ubiquitin-like conjugation systems act in expansion and closure of autophagosome membranes. The ubiquitin-like protein Atg12 is linked to Atg5 by the E1-like activating enzyme Atg7 and E2-like conjugating enzyme Atg10. Atg12-Atg5 then associates with Atg16L (Atg16-like) to form a multimeric complex. In the second conjugation reaction, another ubiquitin-like molecule LC3 (microtubule-associated protein 1 light chain 3) is processed by the cysteine protease Atg4 to reveal a C-terminal glycine (LC3 I). LC3 I is cleaved and linked onto phosphatidylethanolamine (PE) by the E1-like Atg7 and the E2-like Atg3, and translocates from cytoplasm to autophagosomal membrane (LC3 II). The translocation of LC3 is used to monitor autophagy induction and the ratio of protein levels of LC3 II to LC3 I is used as an indication of autophagy levels. Atg4 also cleaves the lipidated LC3 II and frees it from the membrane (Kirisako et al., 2000; Satoo et al., 2009). Recent data suggested that the Atg12-Atg5-Atg16L complex is

an E3 ligase for the LC3-PE conjugation (Fujita et al., 2008; Hanada et al., 2007). Conversely, the LC3 lipidation system is required for the formation of Atg16L complex (Fujita et al., 2008; Sou et al., 2008), indicating interconnection of these two systems (Yang and Klionsky, 2010).

Autophagy can function in the non-selective bulk degradation of cytoplasmic contents or in the selective turnover and elimination of specific cellular components. To selectively target substrates to autophagosomes, protein and organelle substrates are conjugated with ubiquitin, while selective autophagic degradation of lipid droplets may use other signals (Komatsu et al., 2007; Oku and Sakai, 2010; Singh et al., 2009a; Wild and Dikic, 2010). Autophagy cargo receptors such as p62 (sequestosome1/SQSTM1) and NBR1 (neighbor of BRAC1 gene1), interact with both the ubiquitin on cargo and with the autophagy machinery through LC3-interaction (LIR) domains. This enables the cargo receptors to recognize and deliver cargo to autophagosomes (Ichimura et al., 2008a; Ichimura et al., 2008b; Kirkin et al., 2009a; Kirkin et al., 2009b). Cargo receptors are degraded along with the cargo and their accumulation, often in large aggregates in the case of p62, is symptomatic of autophagy inhibition (Komatsu et al., 2007)(Figure 1.1).

### **Regulation of autophagy**

The availability of nutrients, growth factors, and hormones as well as stress, regulate autophagy. mTORC1 is a major negative regulator of autophagy. mTORC1 promotes protein synthesis, cell division and metabolism in response to the nutrient, growth factor and hormone availability, while suppressing autophagy. Growth factors such as insulin or insulin-like growth factors (IGFs) activate signal cascades Ras-Raf-MEK (MAP

kinase/ERK kinase)-ERK1/2 (extracellular signal-regulated kinase) and PI3K (phosphoinositide 3-kinase)-Akt. Both signal pathways suppress the upstream repressor of mTORC1, TSC1/2 (tuberous sclerosis complex 1/2), a GTPase-activating protein complex of the mTORC1 activator Rheb (Ras homolog enriched in brain), thereby keeping mTORC1 active and autophagy suppressed in growth factor replete condition. Tumor cells commonly take advantage of the growth-promoting function of mTOR by acquiring activating mutations in PI3K or inactivating mutations in the PI3K signaling antagonist PTEN (phosphatase and tensin homolog) (Guertin and Sabatini, 2005); therefore, suppression of autophagy may constantly occur in some tumors. Dependent on the availability of amino acids, mTORC1 is targeted to lysosomal membranes by the Ragulator (Ras-related GTPases) complex, which is essential for mTORC1 activation (Sancak et al., 2010). The internal energy status of cells impinges on AMPK (AMP-activated protein kinase), which activates autophagy by inhibiting mTORC1 activity when energy is depleted. Stress such as hypoxia activates AMPK and TSC1/2 complex and induces autophagy. Hypoxia also exerts its effect on autophagy through the hypoxia-inducible factors (HIFs)-mediated transcription of Bnip3, which interacts with and inhibits Rheb (Efeyan and Sabatini, 2010; Neufeld, 2010) (Figure 1.2). Thus, through inhibition of autophagy, mTORC1 coordinates cell growth and catabolism in response to extracellular stimuli.

Autophagy is also regulated by mTORC1-independent signaling. Lowering inositol or IP<sub>3</sub> (inositol-1,4,5-triphosphate) induces autophagy. The physiological relevance that IP<sub>3</sub> regulates autophagy is still unclear. IP<sub>3</sub> receptor was shown to bind Beclin1, which suppresses autophagy (Vicencio et al., 2009). Nevertheless, how IP<sub>3</sub> exactly suppresses



autophagy remains elusive. In skeletal muscle, Akt represses autophagy independent mTORC1 by phosphorylating and inhibiting FOXO (forkhead box O) transcription factors, which control the transcription of *atg* genes (Mammucari et al., 2007), likely providing a tissue specific mechanism of autophagy regulation. Hypoxia also induces autophagy in an mTOR independent manner through HIFs-mediated transcription by inducing Bnip3 (Bcl-2/adenvirus E1B 19-kDa interacting protein 3) to relieve the inhibition of Bcl-2 on Beclin1 (Mazure and Pouyssegur, 2010). Additionally, ammonia derived from glutamine metabolism (glutaminolysis) acts as an autocrine or paracrine factor in regulating autophagy (Eng et al., 2010) (Figure 1.2). Therefore, the metabolite or metabolism of cells also regulates autophagy.

**Figure 1.2. The regulatory pathways of autophagy.**

Autophagy is regulated by various inputs, including growth factors, nutrient availability, hypoxia and energy depletion. Lines with an arrowhead or a cross short line represent positive or negative regulation respectively. Stimulation or inhibition of autophagy is shown in green or red respectively. PTEN is a 3' phosphoinositide phosphatase that counteracts the phosphoinositide 3'phosphorylation activity of PI3K. Redd1 (regulated in development and DNA damage 1) regulates mTORC1 in response to hypoxia (Brugarolas et al., 2004). It is also reported as a transducer of energy depletion and a transcriptional target of HIF (Shoshani et al., 2002; Sofer et al., 2005). IP<sub>3</sub> can cause increase of cytosolic Ca<sup>2+</sup> level by binding to its ER resident receptor IP<sub>3</sub>R (Renna et al., 2010). PIP<sub>2</sub>: phosphoinositol biphosphate.

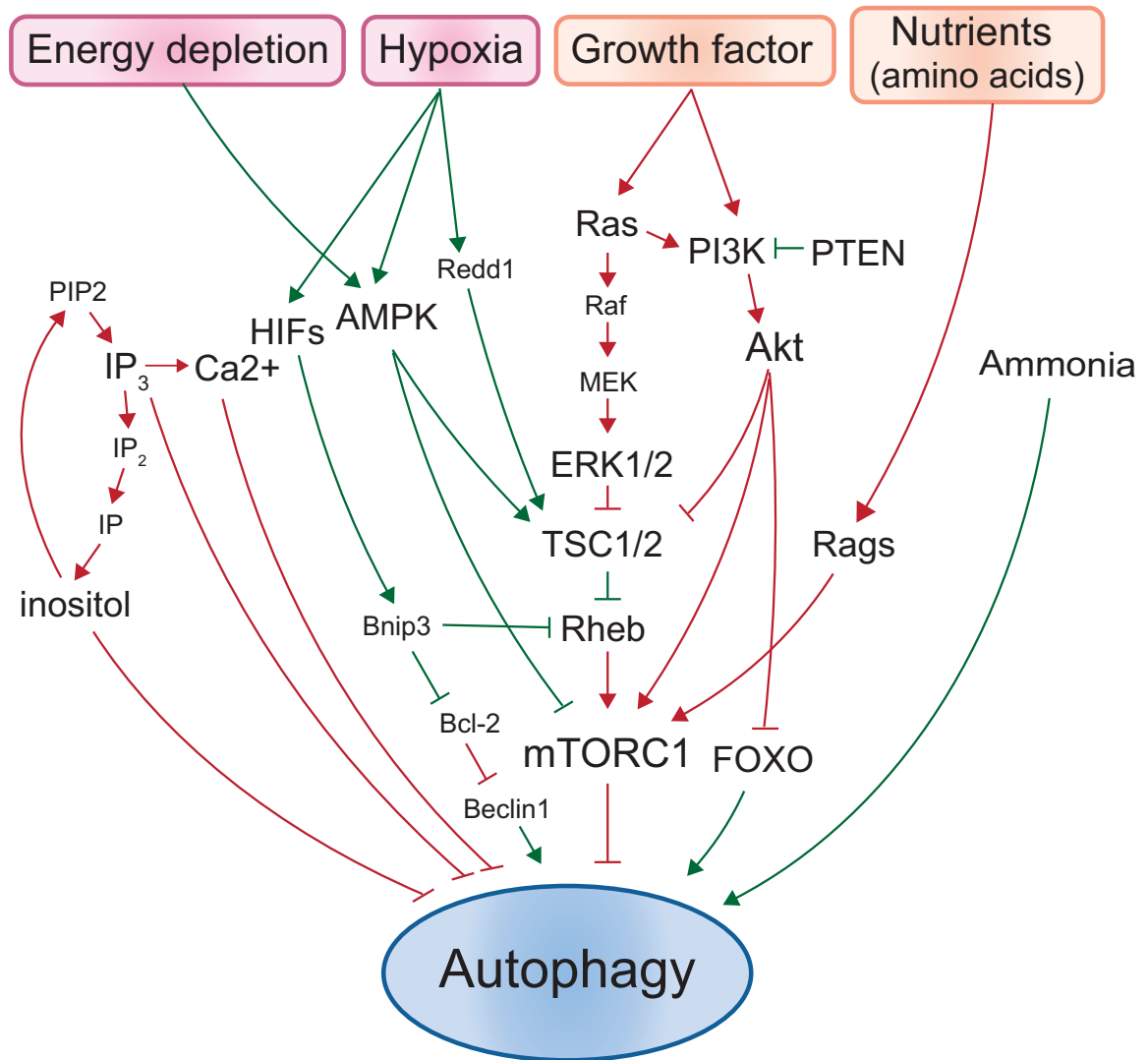


Figure 1.2 The regulatory pathway of autophagy

## **Role of autophagy in physiology**

### *Autophagy in cellular refreshment*

Autophagy-mediated protein quality control. Autophagy constitutively degrades excess or damaged proteins and organelles through its basal activity, which is critical to maintain cellular homeostasis and function. This is especially important for post-mitotic cells, which cannot dilute cellular waste products through cell division. Impaired autophagy in mice causes quiescent cells such as neurons and hepatocytes, to accumulate ubiquitin- and p62-positive protein inclusions, aberrant membranous structures and deformed mitochondria, accompanied by neuronal degeneration and liver injury (Hara et al., 2006; Komatsu et al., 2005). p62- and ubiquitin-containing inclusions (Mallory-Denk bodies) have been associated with a variety of liver diseases including hepatitis and hepatocellular carcinoma (HCC) (Zatloukal et al., 2007). This accumulated p62 causes hepatotoxicity in liver, since genetic ablation of p62 partially suppresses the formation of protein inclusions and liver injury in autophagy-deficient mouse cells (Komatsu et al., 2007). It will be of interest to see if genetic impairment of autophagy is the cause of liver disease in humans and if stimulation of autophagy mitigates disease progression.

In neuronal cells, the accumulation of p62 does not account for neurodegeneration observed in autophagy-deficient mice, since the ablation of p62 did not prevent disease. Failure of autophagy to remove dysfunctional mitochondria (mitophagy) has been mechanistically linked to the neurodegenerative Parkinson's disease (Wild and Dikic, 2010). Additionally, autophagy deficiency accelerates the progression of neurodegenerative diseases precipitated by expression of aggregate-prone mutant proteins, which rely on autophagy for clearance. Enhancement of autophagy has been

suggested as an approach to mitigate neurodegeneration caused by pathogenic proteins such as mutant Huntingtin in Huntington's disease (Renna et al., 2010; Rose et al., 2010) (Figure 1.3).

Autophagy deficit may also contribute to Alzheimer's disease (AD). It is reported recently that presenilin1 (PS1), which is commonly mutated in early-onset familial AD, is required for the acidification of lysosomes/autolysosomes and the clearance of autophagosomes and cargo (Lee et al., 2010). This provides a possibility that defective autophagy causes AD. Restoration of autophagic degradation may slow the progression of AD.

Development of liver disease in  $\alpha$ 1-antitrypsin (AT)-deficient patients exemplifies another case of the significance of autophagy in cellular and organismal well-being by discarding aggregate-prone proteins. A point mutation in the liver-derived secretory AT protein (ATZ) renders it aggregate-prone, causing its accumulation in the ER. ATZ aggregates cause hepatotoxicity, liver injury, inflammation and carcinogenesis. However, only a subgroup of afflicted homozygous patients develop liver fibrosis and eventually hepatocellular carcinoma (Perlmutter, 2009). This discrepancy has been attributed to the interaction of ATZ with genetic or environment determinants. The autophagy-stimulating drug carbamazepine (CBZ) reduces the ATZ load and alleviates the associated symptoms in mice. This suggests that autophagy impairment may predispose ATZ homozygous patients to the ATZ-associated progressive liver disease and that enhancement of autophagy may be beneficial for disease prevention and therapy (Hidvegi et al., 2010) (Figure 1.3).

**Figure 1.3. The interactions between autophagy and diseases.**

Autophagy is involved in the pathogenesis of a variety of diseases. Disorders are shown in blue. Lines with an arrowhead or a cross short line represent positive or negative regulation of the pathway, respectively. Bold or gray lines represent pathways upregulated or downregulated respectively during lipid homeostasis perturbation. The dashed line depicts flux of fatty acids from adipocytes to liver. The asterisk indicates the abnormal deposit of fatty acids in liver when lipid homeostasis is disrupted. The question mark denotes a putative regulation. Yellow shading shows where autophagy is involved in the regulation of lipid homeostasis. Red shading shows inflammation, which promotes tumorigenesis and is suppressed by autophagy. HFD: high-fat diet.

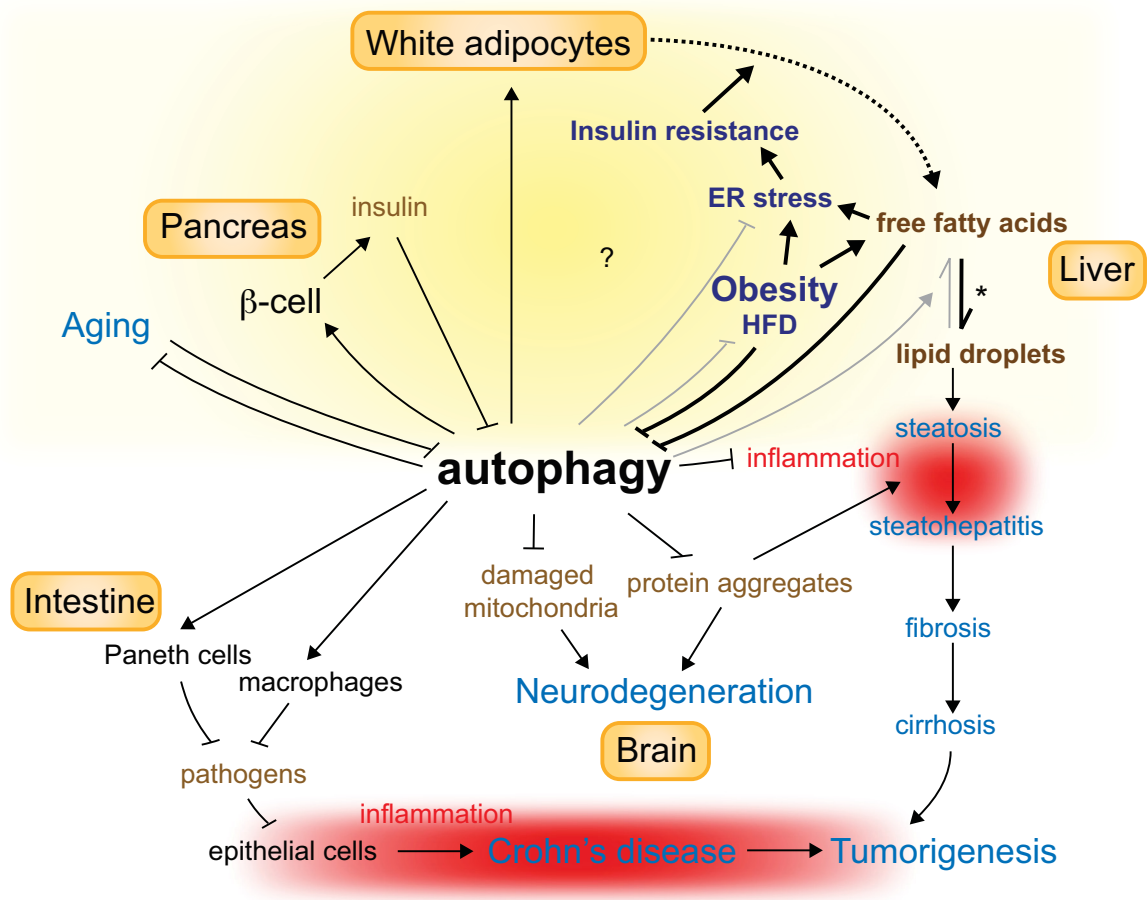


Figure 1.3 The interactions between autophagy and diseases

Autophagy-mediated organelle quality control. Autophagy eliminates damaged organelles and ensures their quality control, thereby maintaining organelle function and preventing the harmful consequences of organelle damage. Autophagy may sustain cellular metabolism through mitochondrial quality control, while impaired autophagy may lead to compromised or altered metabolism in part through the accumulation of dysfunctional mitochondria. Skeletal muscle-specific *atg7* deficiency in mice causes reduced mitochondrial function, revealing the significance of autophagy for preservation of the functional mitochondrial pool (Wu et al., 2009). This mitochondrial function preserved by autophagy is particularly important for cells that need mitochondrial  $\beta$ -oxidation and oxidative phosphorylation for efficient energy production. Moreover, autophagy-mediated turnover of peroxisomes, which also function in fatty acid  $\beta$ -oxidation, might contribute to normal metabolism as well.

The autophagic elimination of damaged proteins and organelles, particularly mitochondria and peroxisomes, also removes potential sources of reactive oxygen species (ROS). ROS production in autophagy-defective mice is associated with tissue damage, chronic cell death and inflammation in the liver. In mouse skeletal muscle, autophagy deficiency causes accumulation of abnormal mitochondria and elevated oxidative stress in myofibers accompanied by muscle degeneration with age (Masiero et al., 2009). Studies in yeast suggest that the peroxisomal autophagy also restricts intracellular ROS. In methylotrophic yeast, autophagy constitutively degrades peroxisomes. Mutations in *atg1*, the yeast homolog of mammalian *Ulk1*, cause peroxisome accumulation and decreased activity of the peroxisomal enzyme catalase, which functions in detoxification and converts  $H_2O_2$  to  $H_2O$ , and increase ROS levels. Senescent human cells also show



accumulation of peroxisomes with reduced capacity to import catalase and increased load of ROS (Legakis et al., 2002; Manjithaya et al., 2010). The autophagy status was not assessed in these aging cells; nevertheless, it is possible that autophagy also preserves the functional integrity of peroxisomes and limits ROS production from peroxisomes in mammals (Manjithaya et al., 2010). Thus, failure of organelle quality control by autophagy can lead to toxic ROS production and disease.

#### *Autophagy in cellular remodeling and development*

Recent studies indicate that autophagy plays a significant role in cellular remodeling and contributes to cell differentiation and development. How autophagy exactly facilitates the cellular rearrangement process remains unclear. Autophagy-mediated non-selective bulk degradation and selective elimination of specific cellular components such as organelles, structural proteins and regulatory factors may both involve in this process. During development, the autophagic protein Atg5 is required for mouse embryos to develop beyond the four-cell to eight-cell stages (Tsukamoto et al., 2008). Role of autophagy in this preimplantation development is still not known. It is likely that autophagy provides building blocks for zygotic synthesis by degrading maternal mRNAs and proteins. Autophagy may, in addition, permit activation of zygotic mRNA and protein synthesis by removing maternal suppressors of zygotic synthesis (Mizushima and Levine, 2010).

Autophagy is also important for differentiation. In some cases, this activity is, at least in part, ascribed to the autophagic elimination of mitochondria. Mice with hematological *atg7* deficiency show severe anemia, and the erythroid cells there from are impaired in mitochondrial elimination and defective in erythroid cell maturation (Mortensen et al.,

2010). B- and T-lymphocyte differentiation also needs autophagy. Mice with hematopoietic cell-specific deletion of *atg7* show decreased B- and T-lymphocyte counts. During normal development of T-cells, mitochondrial content is decreased. T-cells from these mice exhibit accumulation of mitochondria and increased susceptibility to apoptosis (Mortensen et al., 2010). Unlike T-cells, B-cells do not undergo mitochondrial elimination during differentiation. The underlying mechanism of B-cell defects in these mice of hematopoietic-specific autophagy deficiency is still elusive (Mizushima and Levine, 2010). Adipocyte differentiation is another example in which autophagy is required for effective removal of cytosolic components to facilitate the cellular remodeling. During adipogenesis, autophagy is necessary for lipid accumulation and formation of a single large lipid droplet in white adipocytes. Autophagy-deficient white adipocytes contain multilocular lipid droplets and increased mitochondria (Singh et al., 2009b; Zhang et al., 2009). Whether defective mitochondrial clearance is a cause of the impaired differentiation of white adipocytes remains to be investigated.

Recently, autophagy is reported mediating the acquisition of the oncogene-induced senescence phenotype (Young et al., 2009). When senescence occurs, cells undergo substantial remodeling and acquire a phenotype of stable cell cycle arrest but still metabolic active. How autophagy facilitates the remodeling and the establishment of senescent phenotype is still not known. It is possible that autophagy modifies cellular proteome and promotes this process.

#### *Autophagy in metabolic homeostasis*

Recent findings have uncovered multiple roles of autophagy in lipid homeostasis, defects in which can lead to metabolic disorders and fatty liver disease. Fatty liver ranges

from simple steatosis to steatohepatitis, cirrhosis and ultimately can lead to HCC. Dysregulation of lipid metabolism or hepatic lipotoxicity is thought to trigger inflammation and fibrogenesis, which are associated with development of aggressive disease (Trauner et al., 2010). Accumulation of fatty acids and the resulting fatty acid metabolites in liver renders hepatocytes more susceptible to injury, leading to cell death and activation of the inflammatory responses (Neuschwander-Tetri, 2010). This is similar to the phenotypes of autophagy-deficient mice; allelic loss of *beclin1* causes steatosis, steatohepatitis, and spontaneous HCC (Mathew et al., 2009; White et al., 2010). Although it is still unclear how exactly autophagy deficiency contributes to initiation or progression of disease, failure of lipid homeostasis leading to lipotoxicity and chronic inflammation, in addition to p62 accumulation, is a likely possibility.

Autophagy is important for accessing lipid stores through lipophagy and promoting lipid breakdown and preventing deposition in liver (Singh et al., 2009a). In liver, lipid droplets continually undergo hydrolysis and the resulting free fatty acids are used for  $\beta$ -oxidation or synthesis of very low-density lipoprotein (VLDL) for export, or are re-esterified back to triglycerides. Autophagy constitutively degrades lipid droplets (lipophagy) and inhibition of autophagy causes accumulation of lipid droplets in cultured hepatocytes and in mouse liver. Thus, autophagy facilitates the disposal of fatty acids and prevents fatty acid accumulation, which causes lipotoxicity, in liver.

Lipid homeostasis also involves the whole body storage and mobilization of fatty acids, and autophagy contributes to this systemic aspect. Recent data demonstrates that autophagy is required for differentiation of white adipose tissue, where most lipids in the body are stored. Adipocyte-specific *atg7* deficiency in mice causes decreased numbers of

white adipocytes, lower fatty acid levels in plasma and higher insulin responsiveness. These mice are lean even when subjected to a high-fat diet, indicative of altered lipid metabolism (Singh et al., 2009b; Zhang et al., 2009). Moreover, autophagy is also important for the integrity and function of the pancreatic  $\beta$ -cells, which secrete insulin, a hormone regulating the storage and utilization of glucose and fat.  $\beta$ -cell mass and insulin secretion is also reciprocally regulated by fatty acids. Autophagy is again indispensable for the compensatory  $\beta$ -cell mass augmentation in response to a high-fat diet (Ebato et al., 2008; Jung et al., 2008). Therefore, autophagy is involved in local hepatocyte lipid metabolism as well as the systemic regulation of lipid homeostasis. To delineate the impact of autophagy defects in each aspect on pathology of lipid imbalance, inducible conditional knockout mouse models will be useful.

Autophagy is critical for lipid homeostasis. Unfortunately, factors that disrupt lipid homeostasis commonly suppress autophagy, leading to a vicious cycle. To achieve homeostasis, lipophagy should be upregulated during fasting to facilitate the mobilization of fatty acids and  $\beta$ -oxidation in hepatocytes in response to fluctuation in lipid levels. However, a long-term high-fat diet impairs the selectivity of autophagic degradation toward lipid droplets in liver when the high-fat fed mice were starved (Singh et al., 2009a). Obesity also suppresses autophagy. In liver, inhibition of autophagy is likely responsible for the obesity-induced ER stress and insulin resistance (Yang et al., 2010a). Insulin resistance, which mimics starvation, causes flux of fatty acids from white adipose tissue to liver, where it deposited. Impaired lipid autophagy impedes access of lipid. Autophagy inhibition caused insulin resistance further augments the accumulation of lipids in liver. Inhibition of autophagy by a high-fat diet or obesity, therefore, exacerbates

the imbalanced homeostasis (Figure 1.3). In this regard, enhancement of autophagy would help restore lipid homeostasis.

Autophagy declines with age (Bergamini et al., 2004; Levine and Kroemer, 2008; Martinez-Vicente and Cuervo, 2007), correlating with altered metabolism manifested as ectopic fat deposition and intracellular garbage accumulation. This suggests that the imbalance of lipid homeostasis as well as waste accumulation and cellular functional degeneration due to suppressed autophagy may accelerate aging. In worms, autophagy is required for lifespan extension provided by dietary restriction (Jia and Levine, 2007; Levine and Kroemer, 2008). Resveratrol, which mimics starvation and induces autophagy, increases survival of mice on a high-fat diet and reverses age-related syndromes (Baur et al., 2006). Pharmacological inhibition of mTOR by rapamycin also extends lifespan in mice (Harrison et al., 2009), providing a possibility that autophagy improves fitness and mediates longevity in mammals. Rapamycin prolongs the lifespan of mice, even late in their life (Harrison et al., 2009), suggesting that enhancement of autophagy may possibly interrupt aging and related syndromes (Figure 1.3).

#### *Autophagy confronts stress and environmental insults*

Autophagy is upregulated in response to stress, including growth factor and nutrient limitation, energy depletion and hypoxia. In yeast, starvation induces autophagy, which recycles intracellular constituents to support metabolism, leading to adaptation and survival. In mammals, this self-cannibalistic function is conserved, and autophagy deficient mice cannot survive the neonatal starvation period and show indications of energy depletion (Kuma et al., 2004).

The capability of autophagy to degrade proteins, lipids, glycogen and nucleic acids provides cells the flexibility to utilize intracellular components or various stores of nutrients for energy production and biosynthesis under stress (Kuma and Mizushima, 2010; Rabinowitz and White, 2010). Autophagy generates substrates such as nucleosides, amino acids, fatty acids and sugars from the breakdown of intracellular components. Metabolism of different substrates can produce unequal redox equivalents such as NADPH, which can support lipid biosynthesis and maintain cytosolic redox equilibrium (Noguchi et al., 2009; Vander Heiden et al., 2009). Therefore, through selective autophagy, central metabolism may be supported by different substrates to restore metabolic homeostasis in redox status and in biosynthesis in addition to in energy, leading to adaptation. Considering the nature of autophagy: inducible, selective or non-selective degradation in bulk within defined, membrane-enclosed area with enzymes, autophagy is an efficient way to reallocate intracellular “macromolecular stores” to support bioenergetics and for use as building blocks for biosynthetic pathways under stress conditions.

#### *The importance of controlling p62 levels by autophagy*

The capability of autophagy to selectively eliminate specific proteins has an important role in cellular function. The autophagic substrate receptor p62, which is induced by stress to facilitate selective autophagic degradation, itself is a substrate of autophagy (Bjorkoy et al., 2005; Ichimura et al., 2008b; Komatsu et al., 2007; Pankiv et al., 2007). p62 contains oligomerization and protein interaction domains and facilitates protein aggregate formation (Komatsu et al., 2007; Moscat et al., 2007) (Figure 1.4). Failure to clear p62 due to impaired autophagy causes liver damage in mice and promotes

tumorigenesis of allografts (Komatsu et al., 2007; Mathew et al., 2009). Thus, autophagic degradation of a specific protein, p62, has a role in preventing disease.

p62 regulates the NF- $\kappa$ B signaling. p62 is required for oncogenic Ras-driven NF- $\kappa$ B activation and lung adenocarcinoma in mice (Duran et al., 2008). By contrast, aberrant p62 accumulation in autophagy-defective cells abrogates NF- $\kappa$ B signaling that may promote tumorigenesis in the liver (Mathew et al., 2009). The role of NF- $\kappa$ B in tumorigenesis in the liver is becoming clear. NF- $\kappa$ B activates prosurvival and proinflammatory gene transcription. Inhibition of NF- $\kappa$ B activation in hepatocytes in the liver causes hepatocyte cell death. Dying cells activate and recruit immune cells (Kupffer cells), causing cytokine and growth factor production and inflammation, which lead to compensatory proliferation and promote tumorigenesis. By contrast, inhibition of NF- $\kappa$ B in Kupffer cells prevents expression of tumor-promoting cytokines and inflammation and suppresses tumor development (Maeda et al., 2005). Therefore, role of NF- $\kappa$ B in tumorigenesis is context dependent. It will be interesting to delineate the interplay between autophagy deficiency-dependent p62 accumulation and NF- $\kappa$ B signaling in tumorigenesis of different tissue types.

#### **Figure 1.4. Role of autophagic regulation of p62 in cell signaling.**

Stress upregulates autophagy and also induces the accumulation of the autophagic cargo receptor protein p62. Autophagy regulates p62 turnover and its protein levels in cells (denoted by an asterisk). Domains of p62 are depicted diagrammatically. PB1: Phox and Bem1p-1. ZZ: zinc finger. TBS: TRAF6 (tumor necrosis factor receptor-associated factor 6) binding sequence. LIR: LC3 interacting region. KIR: Keap1 interacting region. UBA: ubiquitin-associated domain. Numbers represent the corresponding amino acid positions in mouse p62. NLS: nuclear localization signal. NES: nuclear export signal. Brown arrows indicate p62-protein interactions. PB1 is a homo- or hetero-oligomerization domain used for the p62 polymerization or for binding to other proteins containing PB1 domain such as atypical protein kinase C (aPKCs). ZZ domain binds RIP1 (receptor-interacting protein 1), which activates NF- $\kappa$ B and plays a critical role in necroptosis, a programmed form of necrosis. The dash indicates a putative interaction between p62 and ERK (Komatsu et al., 2010; Moscat et al., 2007; Wooten et al., 2005). Role of p62 in NF- $\kappa$ B activity is intricate. It is reported that p62 is necessary for IKK activation (Duran et al., 2008), whereas evidence also supports that p62 accumulation suppresses the canonical NF- $\kappa$ B pathway, leading to the non-canonical NF- $\kappa$ B activation (Mathew et al., 2009). Accumulation of p62 activates Nrf2, while p62 itself is a transcriptional target of Nrf2, creating a positive feedback loop (Jain et al., 2010). Green or red depicts signals occurring when p62 loses or gains of function respectively. The dashed green line shows a putative role of obesity in tumorigenesis (see Figure 1.3). The dashed red line shows a putative consequence of hyperactive Nrf2.





p62 accumulation activates Nrf2-mediated transcription. Recent studies indicate that the autophagy deficiency-dependent p62 accumulation alters the regulation of another signaling pathway, transcription by Nrf2 (NF-E2 related factor 2). Nrf2 is a transcription factor mediating transcription of antioxidant and detoxifying genes. p62 interacts with Keap1 (kelch-like ECH-associated protein 1), an adaptor for the cullin-based E3-ligase that promotes Nrf2 degradation. By sequestering Keap1, p62 accumulation causes activation of the Nrf2-targeted genes (Jain et al., 2010; Komatsu et al., 2010; Lau et al., 2010). These enzymes encoded by these genes metabolize and detoxify environmental carcinogens or endogenous mutagens such as ROS, protecting cells from damage and carcinogenesis. Counter-intuitively, simultaneous ablation of Nrf2, a cytoprotective antioxidant/detoxifying pathway, in mouse liver alleviates the autophagy deficiency-dependent liver injury (Komatsu et al., 2010). Whether this is directly related to the function of Nrf2-targeted genes remains to be investigated. Constitutive activation of Nrf2 is suggested increasing cancer incidence (Kensler and Wakabayashi, 2010). It will be of interest to see if the autophagy-dependent accumulation p62 promotes tumorigenesis through activating Nrf2.

#### *Autophagy and host defense*

Autophagy can also prevent disease by destroying microbial invaders (xenophagy) besides its role in immune cell differentiation. The autophagic endomembrane system delivers viral nucleic acids or microbial antigens to endosomes/lysosomes for induction of interferon or antigen presentation, activating immune responses (Galluzzi et al., 2010; Levine and Kroemer, 2008; Netea and Joosten, 2010). Recent findings indicate that impairment of the host defense function of autophagy cooperates with environmental

factors such as infection to affect development of the chronic inflammatory bowel disease (IBD) Crohn's disease. IBD increases the risk of developing cancer (Danese and Mantovani, 2010).

The etiology of Crohn's disease has been attributed to the interaction of pathogens and genetic factors. A genome-wide study in patients of Crohn's disease identified the association of variants of autophagy genes *ATG16L1* and *IRGM* (immunity-related GTPase family, M) with disease susceptibility (Hampe et al., 2007; Netea and Joosten, 2010; Parkes et al., 2007). *ATG16L1* is recruited to the bacterial entry site by the pattern recognition receptors (nucleotide-binding oligomerization domain 1/NOD1 and NOD2), triggering autophagic degradation of pathogens and antigen presentation. This prevents persistence of pathogens and chronic inflammation (Cooney et al., 2010; Travassos et al., 2010). Moreover, mice engineered to have a hypomorphic mutation in *ATG16L1* develop disease that mimics Crohn's disease. Hypomorphic *ATG16L1* causes an abnormal response to murine norovirus infection in the intestine. In the mutant mice, Paneth cells, the epithelial cells in the small intestine that secrete antimicrobial peptides to protect the intestine, show abnormal granule packaging and secretion and altered transcription profiles (Figure 1.3). If additionally challenged by chemical-induced injury, these mice manifest aberrant cytokine production and inflammation in intestine. These phenotypes resemble what have been seen in patients with Crohn's disease (Cadwell et al., 2010). Autophagy or autophagy-related process in this type of disease, therefore, is multifaceted (Galluzzi et al., 2010; Netea and Joosten, 2010). It will be interesting to see if these *Atg16L* mutant mice are also tumor prone.

#### *Autophagy and tumorigenesis*

Tumorigenesis is a complex multistage process. It includes tumor initiation, promotion, progression to malignancy and metastasis. This process involves profound alteration of cells in terms of growth, proliferation, metabolism, stress tolerance and survival, and interaction with the microenvironment where they grow. Genetic and epigenetic changes initiate and facilitate progression of normal cells toward malignancy, and chronic tissue damage provides a pro-mutagenic environment to accelerate this process. Chronic inflammation creates a cancer-promoting environment to support survival and proliferation of abnormal cells and hastens progression.

Autophagy suppresses tumor initiation by limiting genome mutation. The housekeeping function of autophagy maintains turnover of proteins and organelles and ensures homeostasis and cellular health, preventing disease conditions. In response to stress, autophagy eliminates damaged proteins and organelles. This damage mitigation of autophagy can be important for survival of tumor cells, which are commonly subjected to metabolic stress due to insufficient vascularization. Autophagy-deficient murine cells, although more susceptible to metabolic stress, have evident DNA damage response activation and have an increased frequency of chromosome gains and losses (Mathew et al., 2007b). Autophagy deficient mice are tumor-prone and liver tumors there from, as well as human liver tumors accumulate p62-containing protein aggregates, ER chaperones and activate the DNA damage response (Mathew et al., 2009). Thus, by taking out the garbage, autophagy may suppress genomic instability to limit tumor initiation and progression.

Autophagy may also hinder proliferation of cells with cancer mutations, in addition to limiting genomic mutations in cells. Inhibition of autophagy delays oncogene-induced

senescence (Young et al., 2009). Senescence is believed to be a tumor suppressive mechanism attributed to the lack of proliferation of senescent cells. By facilitating senescence, autophagy limits propagation of oncogenic mutations thereby suppressing tumorigenesis.

Autophagy suppresses tumor initiation and progression by limiting chronic inflammation. Autophagy maintains homeostasis by removing excess or damaged intracellular components and microbial invaders, and by regulating lipid metabolism. This not only restrains damage, including genome instability, but also the subsequent inflammation. In autophagy suppressive conditions, persistence of unresolved damage leads to chronic cell death and inflammation. In liver, and probably other tissues, this can provide a cancer-promoting environment.

Autophagy provides internal resource to support metabolism and mitigates damage, allowing cells to survive stress. Autophagy-deficient murine tumor cells in a background of defective apoptosis, which commonly occurs in tumors, undergo necrosis when subjected to metabolic stress (Degenhardt et al., 2006). Necrosis also occurs in tumor allografts when apoptosis and autophagy are inhibited concurrently and is associated with inflammation. Therefore, autophagy limits genetic instability and inflammation that predisposes tumor initiation, promotion and progression. In this regard, autophagy stimulation may be tumor preventive.

Tumor cells with oncogenic mutations may be more dependent on autophagy for survival. Tumor cells may have differing requirements for autophagy, dependent on the transformation stages and tumor types. With respect to metabolism, autophagy may promote tumor progression by supporting survival and proliferation of cancer cells

especially those of an aggressive tumor that has increased metabolic demand. During cancer progression, cells often activate pathways that lead to changes in metabolism, such as altered or increased nutrient requirements for energy production and biosynthesis. For example, oncogenic Myc causes cells to become addicted to glutamine for energy production (Wise et al., 2008). Activation of oncogenic pathways may also increase metabolic demand due to promotion of cell proliferation and growth. As autophagy is a pathway that helps to confront these extreme situations, cancer cells may require it to meet their particular metabolic demands. In this regard, inhibition of autophagy may suppress tumor progression.

Autophagy inhibition sensitizes tumor cells to cell death. Since autophagy is a stress survival pathway, aggressive cancer cells may become more sensitive to autophagy inhibition. The autophagy inhibitor chloroquine (CQ), which induces lysosomal stress and prevents autophagic degradation, preferentially kills Myc-expressing mouse cells *in vitro* in a p53-dependent manner. CQ impairs spontaneous lymphomagenesis in *Myc*-transgenic mice that model Burkitt's lymphoma and in *atm*-deficient mice that model ataxia telangiectasia also dependent on p53 (Maclean et al., 2008). Thus lysosomal stress and/or autophagy inhibition may serve as a stress to enhance tumor suppression pathways to harness tumorigenesis when cancer mutations exist.

In a p53-deficient, Myc-induced mouse model of lymphoma, inhibition of autophagy promotes cell death and delays the recurrence of tumors following p53 reactivation or alkylating agent treatment (Amaravadi et al., 2007). This suggests that once cancer occurs, tumor cells may rely on the cytoprotective function of autophagy to survive tumor

suppressor reactivation or therapeutic stress that induces cell death and to survive the tumor microenvironment during latency (White and Dipaola, 2009).

### **Modulation of autophagy for disease prevention and therapy**

Given that autophagy is necessary for normal function of physiology and deregulation of autophagy has been associated with various disorders, therapeutic modulation of autophagy has received a great deal of attention. Thanks to the intensive study of autophagy via high throughput screening efforts, a variety of small molecules that modulate autophagy have been discovered (Renna et al., 2010). Lithium and other bipolar disorder drugs exert their negative influence on IMPase (inositol monophosphatase), depressing intracellular inositol and IP<sub>3</sub> levels and stimulate autophagy. Agents that decrease cytosolic Ca<sup>2+</sup> levels also promote autophagy. Unsurprisingly, numerous agents enhance autophagy via suppression of mTORC1 pathway, the master negative regulator of autophagy. The anti-diabetes drug metformin activates autophagy through inhibition of mTORC1 by activating AMPK. Resveratrol activates the histone deacetylase Sirtuin 1, a sensor of NAD<sup>+</sup>/NADH state and induces autophagy by imitating energy shortage (Baur et al., 2006; Morselli et al., 2009). Molecules that directly target mTORC1 kinase activity and activate autophagy include the allosteric inhibitors, rapamycin analogs, and ATP-competitive agents such as Torin1. Dietary modulation also shows efficacy; caloric restriction, which mimics nutrient and energy depletion suppresses mTORC1 activity and upregulates autophagy (Kenyon, 2005; Levine and Kroemer, 2008). The mechanistic link of spermidine and autophagy activation has not defined yet (Eisenberg et al., 2009).

Molecules that restrict autophagy include various Vps34 inhibitors, which suppress Vps34-Beclin1 complex activity and thereby the initiation of autophagosome formation. CQ and its analog hydroxychloroquine (HCQ) prevent lysosome acidification and impede the degradation of autophagosome cargo (Figure 1.1). Many of these molecules have been used in clinic to treat various diseases for a long time, meaning that we have multiple ways to modulate autophagy available presently. Numerous screens of autophagy modulators are still ongoing. Autophagy machinery components with enzymatic activity such as Ulk1, Atg4 and Atg7 are potential targets for drug discovery. The resulting molecules will add flexibility for modulating autophagy in different steps.

## **Conclusion**

As a cell refreshing and metabolism-regulating pathway, autophagy is required for normal operation of cellular and organismal physiology. Autophagy-deficient models in mice reveal the role of autophagy in precluding progression of chronic diseases. In this perspective, autophagy stimulation/restoration underscores a strategy to prevent disease initiation and progression by sustaining normal physiology and handling stress properly. Of note, since autophagy exerts its effects in multiple aspects, effects of autophagy enhancement are highly cellular context dependent. In addition to benefits, the risk of autophagy stimulation should be taken into account (White and Dipaola, 2009). For example, the magnitude and duration of autophagy activation may result in different consequences. Over induction may lead to perturbation of homeostasis, a traffic jam of autophagy or autophagic cell death. Moreover, cancer cells may depend on autophagy for survival; autophagy stimulation may counterproductively protect cancer cells from death,



leading to cancer progression. In contrast to efforts to exploit the cytoprotective nature of autophagy to prevent tumor initiation, inhibition of autophagy may enhance tumor suppression responses or reduce the fitness of cancer cells and promote cell death of existing cancers. Currently, our understanding of role of autophagy in physiology and pathology is still in an early stage. Further investigation of the role of autophagy in different contexts would warrant development of tractable regimens for modulation of autophagy in cancer prevention and therapy.

## **Chapter 2. EXPERIMENTAL PROCEDURES**

### **Cell culture, cell viability assessment and clonogenic assay**

Cells were cultured in DMEM (GIBCO/Invitrogen) containing 10% FBS (Gemini Bio-Products) and 1% Pen-Strep (GIBCO/Invitrogen) at 38.5°C with 8.5% CO<sub>2</sub> (Mathew et al., 2008). To induce metabolic stress (ischemia), cells were placed in glucose-free DMEM containing 10% FBS and incubated with a defined gas mixture containing 1% oxygen, 5% CO<sub>2</sub> and 94% N<sub>2</sub> (GTS-Welco) (Nelson et al., 2004). For starvation, cells growing in multiwell plates were washed twice with phosphate buffered saline (PBS; GIBCO/Invitrogen) and placed in Hank's Balanced Salt Solution (HBSS; GIBCO/Invitrogen). Cell viability was assessed by a trypan blue exclusion-based cell viability analyzer (Vi-CELL; Beckman Coulter, Inc.) and normalized to untreated cells at the time of initiation of starvation. Clonogenic survival assays were performed following normal, starvation conditions or drug treatment by restoring normal growth medium (DMEM + 10% FBS) and the recovered cells were then fixed by methanol and visualized by staining the plates with Giemsa (Sigma-Aldrich).

### **Constructs and protein expression**

pEGFP-p62 and pEGFP expression vectors were gifts from Dr. Terje Johansen, the pmCherry-Parkin expression vector was generously provided by Dr. Richard Youle, and the p-tFL-LC3 tandem-tagged LC3 expression vector was gift from Dr. Tamotsu Yoshimori. FLAG-HA-tagged p62 was generated by fusion of human full-length p62 to the N-terminal epitope tag and cloned into pCDNA3.1 (+) (Invitrogen). To generate rabbit polyclonal anti-p62 antibodies, the full-length human p62 cDNA was cloned into pMal-C2x (NEB) at EcoRI and SalI enzyme cutting sites. MBP-p62 fusion protein was

expressed in BL21 (DE3) *E. coli* by IPTG induction and affinity purified using an amylose column, and then eluted with maltose. Rabbit polyclonal anti-p62 antibodies were generated by Cocalico Biologicals, Inc. using the MBP-p62 fusion protein as antigen.

### **Generation of cell lines**

Immortal baby mouse kidney (iBMK) epithelial cell lines were generated from neonatal C57Bl/6 mouse as previously described (Degenhardt et al., 2002a; Degenhardt et al., 2002b) (Degenhardt and White, 2006; Mathew et al., 2008; Mathew et al., 2007b).

To obtain cells stably expressing H-ras<sup>V12</sup>, *atg5*<sup>+/+</sup> and *atg5*<sup>-/-</sup> iBMK cell lines were co-transfected with the selection marker plasmid pcDNA3.1/Zeo (Invitrogen) and pCGN-HA-Hras<sup>V12</sup> or control vector pCGN followed by zeocin (Invitrogen) selection; *atg7*<sup>+/+</sup>, *atg7*<sup>-/-</sup>, *beclin1*<sup>+/+</sup> and *beclin1*<sup>+/-</sup> iBMK cell lines were transfected with pCGN-HA-Hras<sup>V12</sup> or control vector pCGN followed by hygromycin B (Sigma-Aldrich) selection; *p62*<sup>+/+</sup> and *p62*<sup>-/-</sup> iBMK cell lines were co-transfected by the selection marker plasmid pCMV-BSD (Invitrogen) with pCGN-HA-Hras<sup>V12</sup> or with control vector pCGN followed by blasticidin (GIBCO/Invitrogen) selection. p62 reconstituted cell lines were derived from *p62*<sup>-/-</sup>Hras cells engineered to stably express EGFP-p62 or FLAG-HA-p62 by co-transfecting with pcDNA3.1/Zeo and pEGFP-p62 or pcDNA3.1(+)-FLAG-HA-p62 followed zeocin selection. *atg5*<sup>+/+</sup>Hras-mCherry-Parkin and *atg5*<sup>-/-</sup>Hras-mCherry-Parkin iBMK cell lines were derived from *atg5*<sup>+/+</sup>Hras or *atg5*<sup>-/-</sup>Hras cell lines transfected with pmCherry-Parkin and followed by geneticin selection. *atg5*<sup>+/+</sup>Hras-GFP-RFP-LC3 and *atg5*<sup>-/-</sup>Hras-GFP-RFP-LC3 cell lines were derived from *atg5*<sup>+/+</sup>Hras or *atg5*<sup>-/-</sup>Hras cells by transfecting with p-tFL-LC3 and followed by geneticin (GIBCO/Invitrogen) selection.

D3-EGFP-LC3 cells were obtained by transfecting D3 (Bax and Bak double knockout) cells with pEGFP-LC3 followed by geneticin selection. Bcl-2-expressing *atg7*<sup>+/+</sup> and *atg7*<sup>-/-</sup> iBMK cells were derived through electroporation of pcDNA3.1-hBcl-2, followed by geneticin (GIBCO-Invitrogen) selection. DNA from Bcl-2-expressing cell lines was purified (Qiagen) and subjected to *atg7* genotyping (Komatsu et al., 2005) to confirm the genotype. Human renal carcinoma cell line RCC4 was generously provided by Dr. William Kaelin (Dana Farber Cancer Institute, Cambridge, MA).

### **Antibodies and reagents**

The following antibodies were used for Western blotting (WB), immunohistochemistry (IHC), and immunofluorescence (IF): p62 (antisera raised against MBP- full-length human p62 described above or that purchased from Enzo Life Sciences); Beclin1 (H-300), Tom20 (FL-145) (Santa Cruz Biotechnology, Inc.); ATG5-ATG12 (Cosmo Bio Co); HA (12CA5) (Roche Applied Science);  $\beta$ -actin (Sigma-Aldrich); LC3 and Ub (Ubi-1) (Novus Biologicals); active caspase-3 (Asp175), S6, phospho-S6 (Cell Signaling Technology, Inc.); E1A (M73), p53 (Ab-1),  $\beta$ -actin (Ab-1) (Oncogene);  $\beta$ -catenin (Zymed); Bcl-2 (N-19) (Santa Cruz); and EGFP (Clontech). WB, IHC and IF procedures were performed as previously described (Degenhardt et al., 2002b; Mathew et al., 2009; Nelson et al., 2004). CCI-779 (Temsirrolimus/Torisel) was obtained from Wyeth/ Pfizer Inc. and chloroquine disulfate salt (CQ), bafilomycin A1, Necrostatin-1, N-acetyl-L-cysteine (NAC), methyl-pyruvate, staurosporine and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were purchased from Sigma-Aldrich.

### **Electron Microscopy**

Tumors from *atg5<sup>-/-</sup>*, *atg7<sup>-/-</sup>*, *beclin1<sup>+/-</sup>*, *p62<sup>-/-</sup>* or wild type control iBMK cells expressing H-ras<sup>V12</sup> were excised at day 11 post-injection and fixed in 0.1M cacodylate buffer with 2.5% glutaraldehyde, 4% paraformaldehyde, and 8μM CaCl<sub>2</sub> and analyzed by a JOEL 1200EX electron microscope as described previously (Degenhardt et al., 2006).

### **Autophagy and mitophagy assessment**

For quantitation of autophagy induction, cells were transiently transfected (Lonza) with the p-tFL-LC3 reporter and allowed to recover overnight. Cells were treated with 1nM bafilomycin A1 to facilitate visualization of autophagosomes and incubated with HBSS for the indicated times prior to fixation and mounting. Images were analyzed at 60x. A minimum of 200 cells was scored for each condition in three replicate experiments. The percentage of cells expressing RFP-LC3 puncta is indicated. The percentage of cells with mCherry-Parkin translocation was quantified by determining the number of cells displaying punctate (rather than diffuse) fluorescence out of a population of 100 fluorescent cells replicated in three independent experiments.

### **Tumor growth assays**

Tumor formation assays and *in vivo* LC3 translocation were performed as previously described (Mathew et al., 2009; Mikhailov et al., 2003; Nelson et al., 2004). Briefly, three mice per cell line and two independently derived cell lines per genotype were used to assess tumor formation. 10<sup>7</sup> (10<sup>6</sup> in Figure 3.4D) cells were subcutaneously injected in nude mice and growth was monitored at the indicated time points. Tumor formation assays were performed using Institutional Animal Care and Use Committee approved protocols.

### **Drug administration in mouse allografts**

$10^7$  D3-EGFP-LC3 cells were subcutaneously inoculated to nude mice and allowed tumor to grow until  $200\text{ mm}^3$ . Tumor bearing mice were sorted into four groups with 4 mice in each group. Vehicle or CCI-779 (10mg/kg/d, 5d/week, 3 cycles) or CQ (60mg/kg/d, 7d/week, 3 cycles) alone or in combination was administrated intraperitoneally. CCI-779 was prepared in diluent and diluted in PBS. CQ was prepared in PBS.

### **Histology**

For paraffin sections, tumor were fixed overnight in 10% buffered formalin solution (Formalde-Fresh, Fisher Scientific), changed to 70% EtOH, then stored and sectioned. For fluorescent localization of p-tFL-LC3 tandem-tagged LC3 in vivo, tumors were fixed overnight in 10% buffered formalin solution followed by dehydration, first in 15% sucrose/PBS for 6 hr and then in 30% sucrose/PBS solution overnight, and sectioned.

### **Energy charge analysis by LC-MS**

Measurements of ATP, ADP or AMP pool size by LC-MS were performed as described previously (Munger et al., 2006). Briefly, cells were cultured in complete media (DMEM, 10% dialyzed FBS, 1% Pen-Strep) for 20-24 hours after which media was replaced with HBSS for 4 hours. Metabolism was quenched by removing the media and adding 4mL of methanol:water (80:20) at  $-80^{\circ}\text{C}$ . Extracted metabolites were dried under nitrogen flow, reconstituted in 350 $\mu\text{L}$  water and measured by LC-MS. Individual pool sizes of ATP, ADP or AMP were normalized to cell number and to known amounts of uniformly  $^{15}\text{N}$ -labeled ATP (4.89nmols/mL), and  $^{13}\text{C}$ -labeled ADP (2.27 nmols/mL) and AMP (0.35 nmols/mL), added to the extraction media. Statistical significance was calculated by 2-way ANOVA with Bonferroni posttest.

**Assessment of mitochondrial membrane potential and ROS levels**

Live cells were stained with 25nM MitoTracker Red CMXRos (Molecular Probes/Invitrogen) and 25nM MitoTracker Green FM (Molecular Probes/Invitrogen) fluorescence dyes for 30 minutes under growth conditions, washed with growth medium and analyzed by flow cytometry (BD influx cell sorter; BD Biosciences). ROS levels were determined using 10  $\mu$ M 2'-7'-dichlorodihydrofluoresceine diacetate (DCF-DA, Molecular Probes) as described previously (Mathew et al., 2009). Mitochondria superoxide was detected with MitoSOX Red fluorogenic dye as the manufacturer recommended (Molecular Probe).

## **Chapter 3. Activated Ras Requires Autophagy to Maintain Oxidative Metabolism and Tumorigenesis**

### **SUMMARY**

Macroautophagy (autophagy) is a catabolic survival pathway utilized by cells to support metabolism in response to starvation and to clear damaged proteins and organelles in response to stress. Autophagy captures and delivers intracellular components to lysosomes where they are degraded and recycled. Tumor cells utilize autophagy to survive in poorly vascularized, hypoxic regions suggesting that autophagy inhibition may enhance the efficacy of cancer therapy. We report here that expression of an activated H-ras<sup>V12</sup> oncogene upregulates basal autophagy, and that autophagy is required for viability of Ras-expressing tumor cells in starvation and tumorigenesis. In Ras-expressing cells, defective autophagosome formation or cargo delivery causes accumulation of abnormal mitochondria with a reduced functionality as revealed by failure in maintaining mitochondrial membrane potential during starvation. Autophagy defects also lead to energy depletion in starvation. As mitochondria sustain viability of Ras-expressing cells in starvation, autophagy maintains the pool of functional mitochondria necessary to support growth of Ras-expressing tumors. This “autophagy addiction” suggests that therapeutic strategies to inhibit mitochondrial function or autophagy hold potential in treatment of cancers with activated Ras.



## INTRODUCTION

The products of essential autophagy genes (*atg*) (*atg5*, *atg6/beclin1*, *atg7* and *lc3/atg8*, among others) respond to starvation, forming double membrane phagophores that engulf cellular proteins, lipids and organelles, capturing them in autophagosomes that are degraded in lysosomes (Levine and Kroemer, 2008). This catabolic cellular self-degradation provides a mechanism to produce building blocks for macromolecular synthesis and to maintain energy homeostasis through intracellular recycling in periods of nutrient limitation (Rabinowitz and White, 2010). In established tumors, autophagy localizes to hypoxic tumor regions where it promotes survival, indicating that cancer cells utilize the catabolic function of autophagy to tolerate stress (Degenhardt et al., 2006; Karantza-Wadsworth et al., 2007; Mathew et al., 2007a; Mathew et al., 2009; Mathew et al., 2007b). As autophagy primarily generates substrates for oxidative metabolism, the means by which autophagy promotes survival in hypoxia are not clear. Autophagy also mitigates cellular damage by preventing the toxic buildup of unfolded and aggregation-prone proteins, lipids and damaged organelles. This waste-removal function of autophagy may be important both for cell survival and tumor suppression (Levine and Kroemer, 2008; Mathew et al., 2007a; White et al., 2010). Autophagy defects cause accumulation of p62- and ubiquitin (Ub)-containing protein aggregates, and damaged organelles, particularly mitochondria (Hara et al., 2006; Komatsu et al., 2006; Komatsu et al., 2007; Mathew et al., 2009). This is associated with production of reactive oxygen species (ROS), genome damage and spontaneous tumor development, suggesting that autophagy suppresses chronic tissue damage and cancer initiation (Mathew et al., 2009). As autophagy both mitigates damage and maintains energy homeostasis, we hypothesized

that autophagy has opposing roles in cancer initiation and in established tumors. Whereas damage mitigation may be important for suppressing tumor initiation and for limiting progression of early stage tumors, in aggressive cancers, the increased metabolic demand of cell proliferation and growth in a stressed microenvironment may instead cause dependency on autophagy for survival. We envisioned that the tumor-promoting role of autophagy is two-fold: providing energy substrates during periods of nutrient limitation, and preserving organelle function required for cell growth.

## RESULT

### **Oncogenic H-Ras<sup>V12</sup> elevates the basal level of autophagy**

We tested the hypothesis that activation of a strong cell growth-promoting oncogene such as H-ras<sup>V12</sup> would alter the cellular requirement for autophagy. Specifically, we hypothesized that cell expressing activated Ras would be less able to reduce metabolic expenditure during periods of starvation; accordingly, they might be more dependent on autophagy. An activated H-ras<sup>V12</sup> gene was introduced into immortal, non-tumorigenic baby mouse kidney epithelial (iBMK) cells (Figure 3.1A). Autophagy was measured by the frequency of cells displaying membrane translocation of the autophagosome component reporter LC3, and by assessing proteolytic processing of endogenous LC3-I to LC3-II. In immortal, non-tumorigenic iBMK cell lines without Ras, the level of basal autophagy was low (0-5%) in nutrient replete conditions and was upregulated more than ten-fold by starvation (Hank's balanced salt solution, HBSS) (Figure 3.1B, C). In contrast, expression of H-ras<sup>V12</sup> (Ras) increased basal autophagy ten-fold and limited starvation-induced autophagy (Figure 3.1B, C). Isogenic iBMK cell lines deficient for the essential autophagy genes *atg5* or *atg7* were deficient in autophagy (Figure 3.1B, C) and allelic loss of the essential autophagy gene *beclin1* produced a partial autophagy defect (Figure 3.1B). Accumulation of the autophagy substrate p62 was only observed when autophagy was genetically impaired in vitro (Figure 3.1A) or in vivo (see below), suggesting that overexpression of Ras does not interfere with autophagic flux. This high level of autophagy in Ras-expressing cells occurred despite mTOR activation, suggesting mTOR-independent regulation.

### **Autophagy is required for H-Ras<sup>V12</sup>-expressing cells to survive starvation**

In nutrient replete conditions, autophagy deficiency had no effect on proliferation or survival of Ras-expressing cells (Figure 3.1D, E). In starvation, Ras-expressing, autophagy-competent cells survived, whereas autophagy-deficient cells did not, and loss of viability was accompanied by activation of caspase-3 indicative of apoptosis (Figure 3.1C-E). Starvation sensitivity of autophagy-deficient cells was Ras-dependent, since autophagy status did not differentially effect survival of cells without Ras (Figure 3.1D, E). Autophagy defects did not sensitize cells to the proapoptotic ATP-competitive non-selective kinase inhibitor staurosporine (Figure 3.1F), suggesting that autophagy-dependent survival was specific to metabolic stress. Apoptosis of Ras-expressing, autophagy-defective cells was not rescued by methyl-pyruvate, ROS scavengers (Figure 3.2A), nor was ROS production apparent (Figure 3.2B), suggesting that survival impairment was not merely due to mitochondrial substrate limitation or elevated oxidative stress. Thus Ras expression promotes cellular survival in an autophagy-dependent manner when nutrients are limiting.

**Figure 3.1. Autophagy deficiency sensitizes cells expressing oncogenic H-ras<sup>V12</sup> to starvation.**

- (A) Generation and characterization of H-Ras<sup>V12</sup>-expressing autophagy-competent and -deficient cell lines. HA-tagged H-Ras<sup>V12</sup> was stably expressed in autophagy-competent or -deficient immortal baby mouse kidney (iBMK) cell lines. Protein levels of HA-tagged H-ras<sup>V12</sup>, Beclin1, Atg12-conjugated Atg5 and p62 were analyzed by Western blotting. Expression of H-ras<sup>V12</sup> did not affect Beclin1 levels. *beclin1*<sup>+/-</sup> cells showed decreased Beclin1 expression. In *atg7*<sup>-/-</sup> cells, Atg12 failed to conjugate onto Atg5. The autophagy substrate protein p62 accumulated in autophagy deficient cells.
- (B) Stable expression of H-ras<sup>V12</sup> elevates basal autophagy levels. Autophagy-competent or -deficient cells expressing H-ras<sup>V12</sup> or vector were transiently transfected with p-tFL-LC3 and allowed to recover overnight prior to starvation. Cells were starved (HBSS) for 4 hours in the presence of 1nM Bafilomycin A1 to facilitate visualization of autophagosomes. Representative images show localization of RFP-LC3. Numbers indicate frequency of cells with LC-3 translocation to autophagosomes (punctate localization).
- (C) Autophagy deficiency sensitizes H-ras<sup>V12</sup>-expressing cells to starvation. *atg7*<sup>+/+</sup> and *atg7*<sup>-/-</sup> cells stably expressing H-ras<sup>V12</sup> or vector were treated with HBSS for 12 hours. Cells were harvested at time indicated and analyzed by western blotting. Expression of H-ras<sup>V12</sup> elevated basal autophagy and starvation induced autophagy as indicated by increased LC-3 II to LC-3 I ratio. Autophagy-deficient

cells expressing H-ras<sup>V12</sup> are more sensitive to starvation as indicated by caspase-3 activation.

(D) Autophagy-deficient cells fail to take advantage of oncogenic H-ras<sup>V12</sup> in terms of survival and proliferation under starvation. Cells were kept in normal growth medium (DMEM+10% FBS) or treated with HBSS for indicated time and viability were assessed by trypan blue exclusion. Graphs present relative viability to time 0.

(E) Clonogenic survival of (D). After subjected to starvation, growth medium were restored to allow cells to recovery for 1 day. Cells were then fixed and stained with Giemsa.

(F) The sensitivity of H-ras<sup>V12</sup> cells to staurosporine is not associated with autophagy capability. *atg5*<sup>+/+</sup> or *atg5*<sup>-/-</sup> cells expressing H-ras<sup>V12</sup> were treated with 1 $\mu$ M staurosporine for 24 hours and cell viability was assessed.

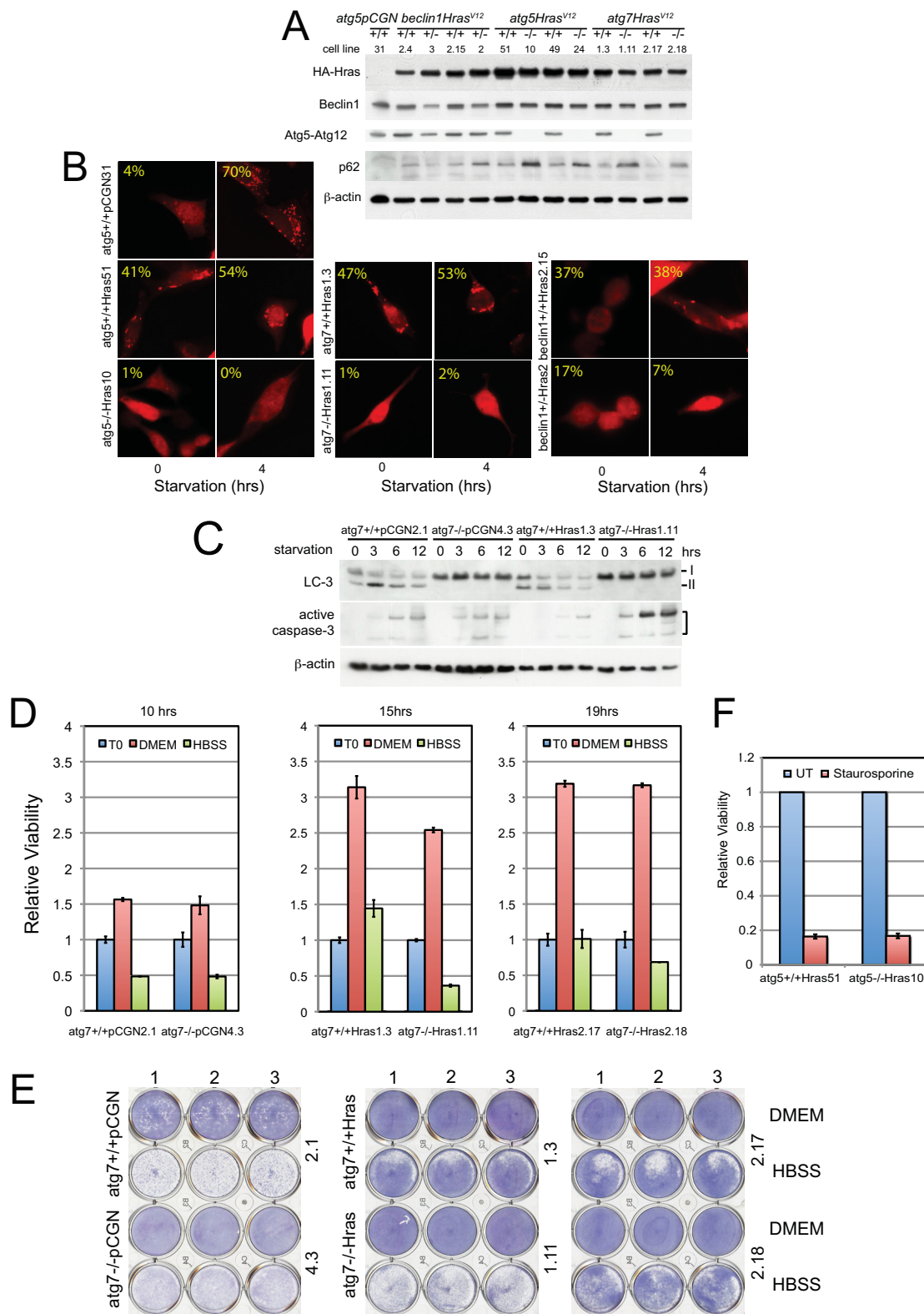


Figure 3.1 Autophagy deficiency sensitizes cells expressing oncogenic H-ras<sup>V12</sup> to starvation

**Figure 3.2. Viability of autophagy-deficient H-Ras<sup>V12</sup>-expressing cells under starvation is not rescued by the mitochondrial metabolic substrate methyl-pyruvate or ROS scavengers.**

- (A) Supplement of metabolic substrate or ROS scavenger does not rescue cell death of autophagy-deficient H-ras<sup>V12</sup> cells in starvation. *atg7<sup>+/+</sup>* and *atg7<sup>-/-</sup>* cells stably expressing H-ras<sup>V12</sup> were treated with HBSS in the presence of 0.5mM of the cell permeable metabolic substrate methyl-pyruvate (MP) or 1mM of ROS scavenger N-acetyl-L-cysteine (NAC) for 15 hours and cell viability was assessed by trypan blue exclusion.
- (B) Autophagy-deficient H-ras<sup>V12</sup> cells do not show apparent ROS production. Overlay showing ROS levels in H-ras<sup>V12</sup>-expressing cells under normal (blue) or 3 hours of starvation (HBSS; red) analyzed by flow cytometry using the ROS sensor 2'-7'-dichlorodihydrofluorescence diacetate (DCF-DA). Numbers indicate mean fluorescence signal for 10<sup>4</sup> cells.



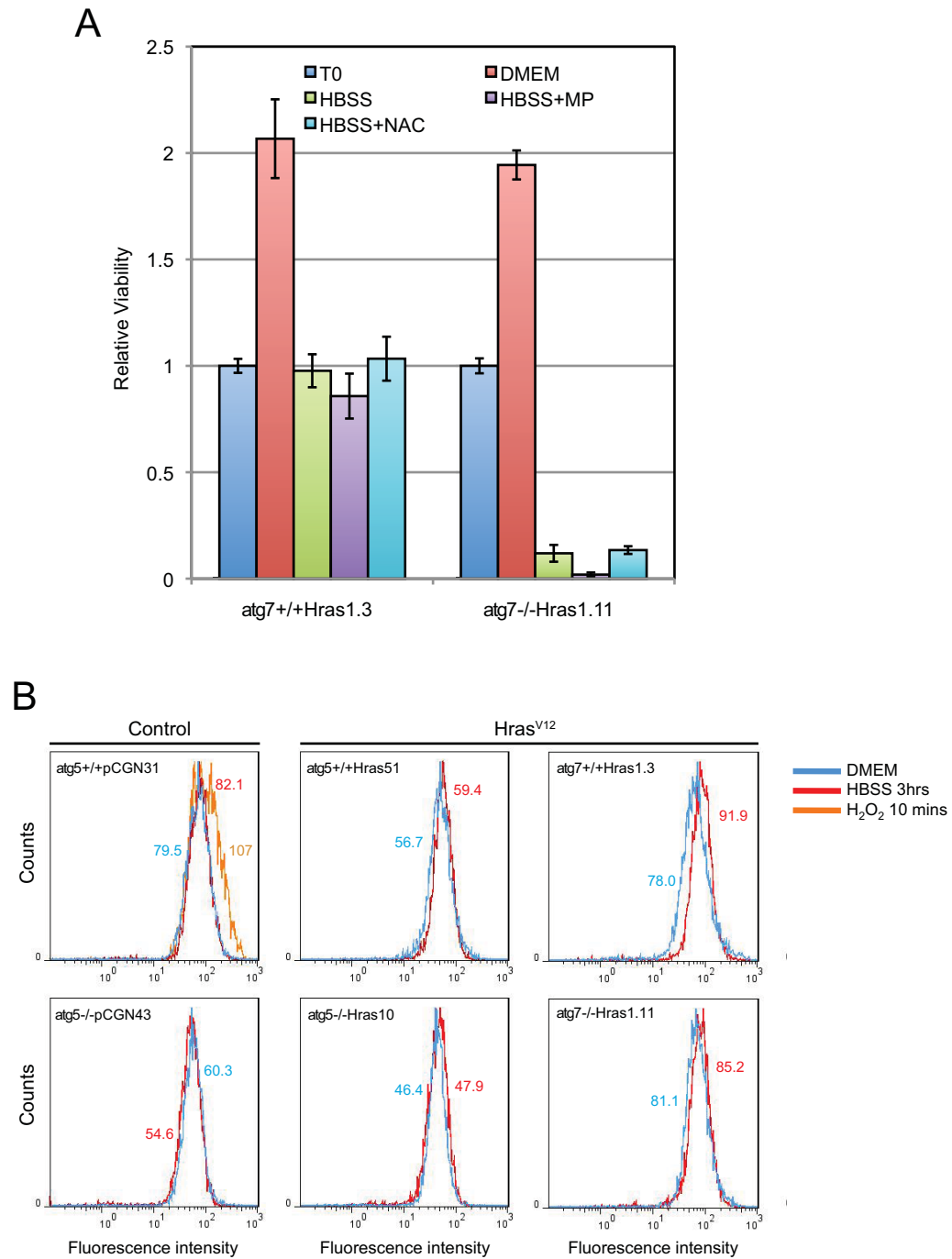


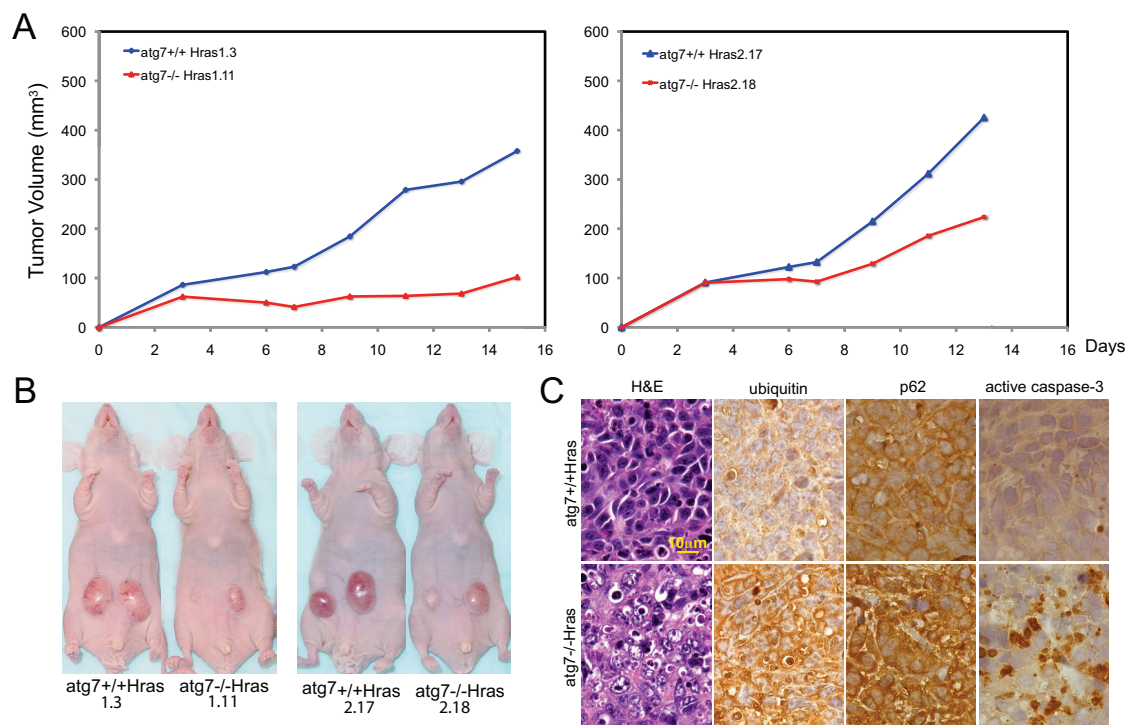
Figure 3.2 Viability of autophagy-deficient H-Ras<sup>V12</sup>-expressing cells under starvation is not rescued by the mitochondrial metabolic substrate methyl-pyruvate or ROS scavengers

**Autophagy deficiency impairs H-ras<sup>V12</sup>-mediated tumorigenesis**

To assess the role of autophagy in Ras-mediated tumorigenesis, non-tumorigenic iBMK cells were transfected with Ras, which dramatically promotes tumor formation (Figure 3.3A, B) (Degenhardt et al., 2006). Ras-expressing *atg7*<sup>-/-</sup> cells displayed reduced tumor growth (Figure 3.3A, B) and tumors displayed abnormal histology, active caspase-3, and p62 and Ub accumulation (Figure 3.3C). Facilitation of tumorigenesis by autophagy is Ras-specific, since growth of tumors without Ras is not reduced by autophagy deficiency (Degenhardt et al., 2006; Karantza-Wadsworth et al., 2007; Mathew et al., 2009; Mathew et al., 2007b).

**Figure 3.3. Autophagy deficiency impairs H-ras<sup>V12</sup>-mediated tumorigenesis.**

- (A) Deficiency in autophagy suppresses H-ras<sup>V12</sup>-driven tumor formation. Two pairs of *atg7*<sup>+/+</sup> and *atg7*<sup>-/-</sup> cells stably expressing comparable levels of H-ras<sup>V12</sup> were subcutaneously injected (10<sup>7</sup> cells/ injection) into nude mice (3 mice /group) and tumor growth was monitored.
- (B) Representative images of mice of (A) at the end of tumor assay showing less tumorigenic of autophagy-deficient Ras-expressing cell lines.
- (C) Representative images of histology and immunohistochemistry (IHC) analysis of tumors from (A). H&E staining shows deterioration and necrosis in *atg7*<sup>-/-</sup>Hras tumors. Immunohistochemistry shows accumulation of ubiquitinated proteins and p62 and increased active caspase-3 indicative of apoptosis in *atg7*<sup>-/-</sup>Hras tumors.



## Genetic ablation of the autophagy adaptor p62 impairs H-ras<sup>V12</sup>-mediated tumorigenesis

p62 binds LC3 and Ub on modified proteins, including those on organelles such as depolarized mitochondria, thereby targeting cargo to autophagosomes for degradation (Geisler et al., 2010; Pankiv et al., 2007). Interestingly, deficiency in p62 impairs spontaneous lung adenocarcinoma development in mice upon activation of an oncogenic K-ras allele (Duran et al., 2008). We tested the hypothesis that p62 deficiency impairs cargo delivery to autophagosomes, thereby compromising Ras tumorigenesis by the same mechanism as deficiency in atg7. Ras-expressing *p62*<sup>-/-</sup> cells (Figure 3.4A) had reduced viability in starvation (Figure 3.4B, C) and decreased tumorigenicity (Figure 3.4D, E, G, H) in comparison to *p62*<sup>+/+</sup> and p62-reconstituted controls. Ras-expressing *p62*<sup>-/-</sup> tumors showed evidence of abnormal histology, apoptosis (active caspase-3) and Ub accumulation (Figure 3.4F, I) indistinguishable from Ras-expressing *atg7*<sup>-/-</sup> tumors. Thus, interfering with either autophagosome cargo delivery or autophagosome formation has the common feature of impeding Ras-dependent tumorigenesis.

**Figure 3.4. Deficiency of the autophagy cargo shuttling factor p62 impairs H-ras<sup>V12</sup>-mediated tumorigenesis.**

- (A) Reconstitution of p62 in H-Ras<sup>V12</sup>-expressing *p62*<sup>-/-</sup> cells. EGFP-tagged- or FLAG-HA-tagged-p62 were stably expressed in H-ras<sup>V12</sup>-expressing *p62*<sup>-/-</sup> iBMK cell lines. Protein levels of endogenous p62 in *p62*<sup>+/+</sup> Ras-expressing cells, and reconstituted EGFP-p62 and FLAG-HA-p62 in *p62*<sup>-/-</sup> Ras-expressing cells were analyzed by Western blotting.
- (B) Re-expression of p62 rescues the starvation-induced cell death of *p62*<sup>-/-</sup>Hras cells. *p62*<sup>-/-</sup>Hras cells with reconstituted p62 or control vectors were treated with HBSS for 16 hours and assessed for viability by trypan blue exclusion. Cells viability was relative to time 0.
- (C) Clonogenic survival of (B). After starvation, normal growth medium was restored and cells were allowed to recover for 1 day. Recovered cells were then fixed and stained with Giemsa.
- (D) p62 is required for efficient tumorigenesis driven by Ras. Tumor assay were performed as figure 3.3 (with 10<sup>6</sup> cells/injection) using *p62*<sup>+/+</sup>Hras and *p62*<sup>-/-</sup>Hras cell lines.
- (E) Representative mouse images of (D) at the end of experiment. p62 deficiency impairs Ras-mediated tumorigenesis.
- (F) Histology and IHC analysis of tumor sections from (D). *p62*<sup>-/-</sup>Hras tumors displays accumulation of ubiquitinated proteins, activation of caspase-3 and deteriorated appearance compared to *p62*<sup>+/+</sup>Hras tumors.

- (G) Re-expression of p62 restores the tumorigenicity of H-ras<sup>V12</sup>-expressing *p62*<sup>-/-</sup> cells. Tumor assay on *p62*<sup>-/-</sup>Hras cells re-expressing EGFP-p62 or EGFP control was performed as described in figure 3.3.
- (H) Representative mouse images of (G) at day15. Re-expression of p62 facilitates *p62*<sup>-/-</sup>Hras cells to form tumors.
- (I) Histology and IHC analysis of tumor sections from (G). p62 reconstitution suppresses accumulation of ubiquitinated proteins and caspase-3 activation.

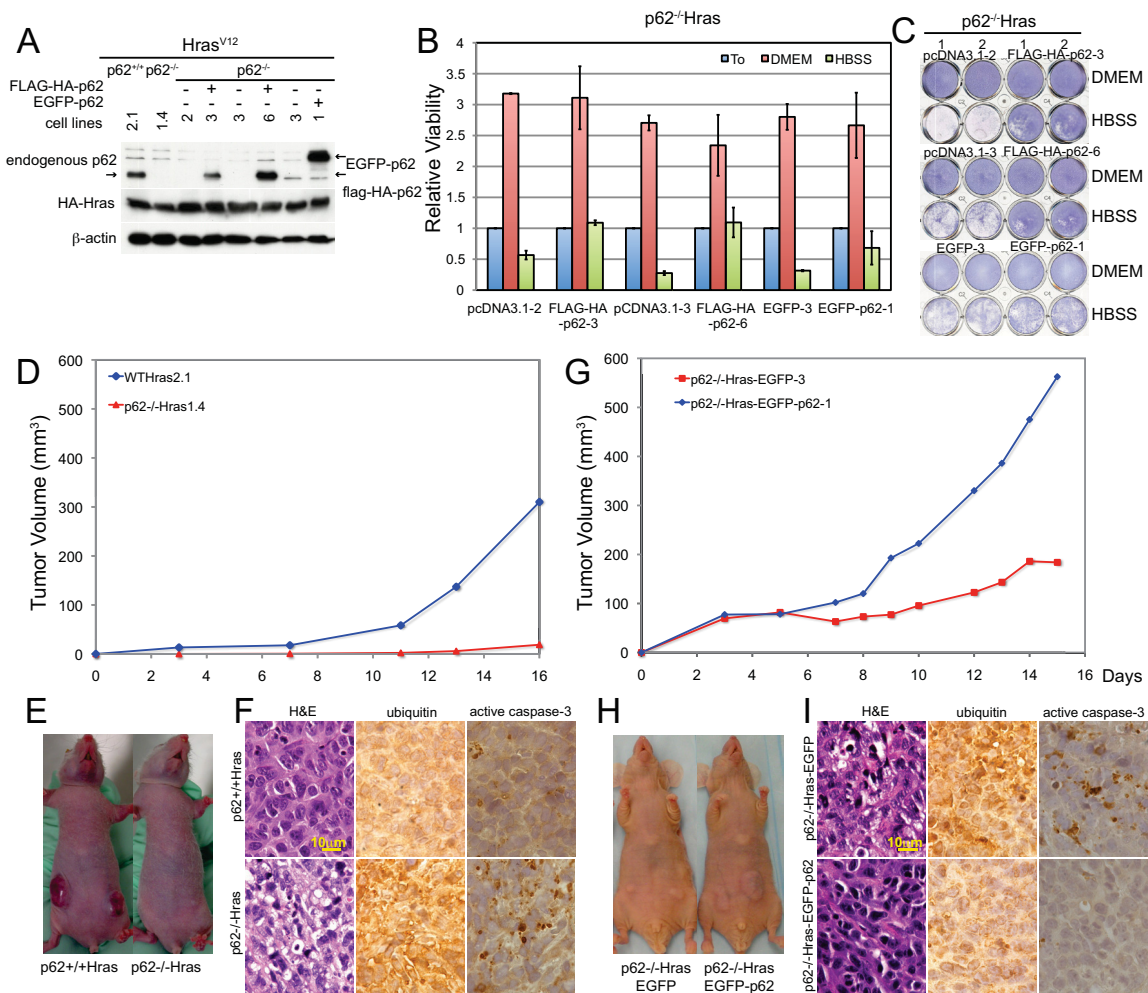


Figure 3.4 Deficiency of the autophagy cargo shuttling factor p62 impairs H-ras<sup>V12</sup>-mediated tumorigenesis



### **Autophagy is indispensable to mitochondrial functionality necessary for Ras-expressing cells to survive starvation**

To begin to address the mechanism by which autophagy defects impair tumorigenesis by Ras, tumors were examined by electron microscopy. In Ras-expressing *atg5*<sup>-/-</sup>, *atg7*<sup>-/-</sup> and *p62*<sup>-/-</sup> tumors, there was striking accumulation of abnormal, swollen mitochondria, suggesting mitochondrial loss of function (Figure 3.5A). To test if autophagy defects impair mitochondrial function, maintenance of mitochondrial membrane potential was examined. When starved, autophagy-defective cells more readily lost membrane potential, consistent with reduced mitochondrial functionality (Figure 3.5B). Accumulation of deformed mitochondria indicated failure of clearance of damaged mitochondria by autophagy. Mitophagy is triggered by mitochondrial depolarization, translocation of the E3 ligase Parkin to mitochondria and ubiquitination of VDAC1, causing p62 binding and autophagosome targeting (Wild and Dikic). To assess mitophagy initiation in starvation, Ras-expressing *atg5*<sup>+/+</sup> and *atg5*<sup>-/-</sup> cells were stably transfected with a tagged-Parkin expression vector and examined pre- and post-starvation for mitochondrial translocation (Narendra et al., 2008). Parkin translocation occurred at low levels in Ras-expressing *atg5*<sup>+/+</sup> and *atg5*<sup>-/-</sup> cells under nutrient replete conditions and was induced by either the mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) or starvation, independent of autophagy functional status (Figure 3.5C). *atg5* deficiency impaired clearance of Parkin following starvation, consistent with defective mitophagy (Figure 3.5D). The failure of autophagy-defective cells to engage mitophagy-mediated degradation of mitochondria in starvation was also

evident from the sustained high levels of the mitochondrial protein Tom20 (Figure 3.5D, E).

Since autophagy recycles internal resource to sustain metabolism during period of nutrient depletion, we next sought if autophagy impacted mitochondrial functionality on supplement of substrates for TCA cycle. To reveal the necessity of autophagy in mitochondrial metabolism, glucose and glutamine, the two main bioenergetic substrates in mammalian cells were specifically deprived from the culture medium. Glucose feeds into glycolysis to produce energy, or the resulting pyruvate further feeds into TCA cycle for mitochondrial oxidative phosphorylation. Glutamine feeds into TCA cycle by glutaminolysis. Deprivation of either one did not show apparent impact on cell viability, indicating that these two substrates compensated each other (data not shown). Simultaneous deprivation of glucose and glutamine caused cell death in autophagy-deficient cells (Figure 3.5F). Ras-expression exacerbated the loss of viability, indicative of elevated metabolic demand. This was rescued by supplying methyl-pyruvate, a cell permeable substrate of mitochondrial oxidative phosphorylation, suggesting that autophagy-mediated internal resource recycling sustained mitochondrial metabolism necessary for cells to survive nutrient depletion conditions (Figure 3.5F).

To test if mitochondrial function was required for viability in starvation, mitochondria were depolarized with CCCP and survival in starvation was assessed. While mitochondrial depolarization had minimum effect on survival of cells in nutrient replete conditions, viability in starvation was abrogated independent of autophagy status (Figure 3.6A). Thus mitochondrial function is critical for survival in starvation.

Given that autophagy deficiency in Ras-expressing tumor cells resulted in defective mitochondrial function, the levels of ATP, ADP, AMP and energy charge (EC) was examined. Basal EC, a measure of the overall energy status of the cell, was comparable in both autophagy-competent and -defective cells with and without Ras. Ras expression resulted in a drop in EC in *atg5<sup>+/+</sup>* cells upon starvation, which was decreased further by autophagy deficiency (Figure 3.6B). Although mitochondrial ROS production has been associated with Ras-driven tumorigenesis by Ras (Weinberg et al., 2010) data regarding EC implicate the energy-generating function of mitochondria. Ras expression amplifies energy depletion in starvation, rendering cells autophagy dependent to buffer this demand through preservation of mitochondrial functionality by maintaining functional pool of mitochondria and also by production of catabolically derived metabolic substrates. In autophagy-defective cells this metabolic insufficiency in starvation produces an acute energy crisis leading to cell death.

**Figure 3.5. Autophagy is required to maintain mitochondrial functionality for Ras-expressing cells to survive stress.**

- (A) Electron micrographs showing accumulation of abnormal swollen mitochondria in *atg5*<sup>-/-</sup>, *atg7*<sup>-/-</sup> and *p62*<sup>-/-</sup> Hras tumors. Arrows indicate mitochondria. Inset: representative mitochondria in higher magnification. Scale bars represent 500nm.
- (B) Starvation causes loss of mitochondrial membrane potential that is more evident in autophagy-deficient H-ras<sup>V12</sup> cells. Autophagy-competent (*atg5*<sup>+/+</sup> or *atg7*<sup>+/+</sup>) or -deficient (*atg5*<sup>-/-</sup> or *atg7*<sup>-/-</sup>) cells stably expressing H-ras<sup>V12</sup> along with cells expressing vector were treated with HBSS for indicated time and probed for total mitochondria with MitoTracker Green FM and for mitochondria maintaining membrane potential with MitoTracker Red and analyzed by flow cytometry.
- (C) Dissipation of mitochondrial membrane potential by the uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) or starvation causes parkin translocation. *atg5*<sup>+/+</sup>Hras and *atg5*<sup>-/-</sup>Hras cells stably expression mCherry-Parkin were treated with CCCP or HBSS for 4 hours and assessed for mCherry-Parkin localization. Numbers indicate frequency of cells with punctate mCherry-Parkin.
- (D) Turnover of parkin is impaired in autophagy-deficient cells, indicative of mitophagy deficiency. *atg5*<sup>+/+</sup>Hras or *atg5*<sup>-/-</sup>Hras cells stably expressing mCherry-Parkin were treated with HBSS for 7 hours and immuno-probed for the mitochondrial protein Tom 20. Numbers denote frequency of mCherry-Parkin positive cells.
- (E) Western blot showing down regulation of Tom 20 during starvation exclusively in autophagy-competent cells. *atg5*<sup>+/+</sup>Hras, *atg5*<sup>-/-</sup>Hras, *atg7*<sup>+/+</sup>Hras and *atg7*<sup>-/-</sup>Hras

cells were treated with HBSS. Cell extracts were harvested at time indicated and subjected to western blot analysis.

- (F) The susceptibility of autophagy-deficient cells to glucose and glutamine double deprivation is reversed by the mitochondrial metabolic substrate methyl-pyruvate. *atg7<sup>+/+</sup>*Hras and *atg7<sup>-/-</sup>*Hras cells were grown in DMEM deprived of glucose and glutamine in the absence or presence of 1mM methyl-pyruvate for 33 hours and allowed to recover in regular medium for 1 day. Clonogenic survival was assessed by Geimsa staining.

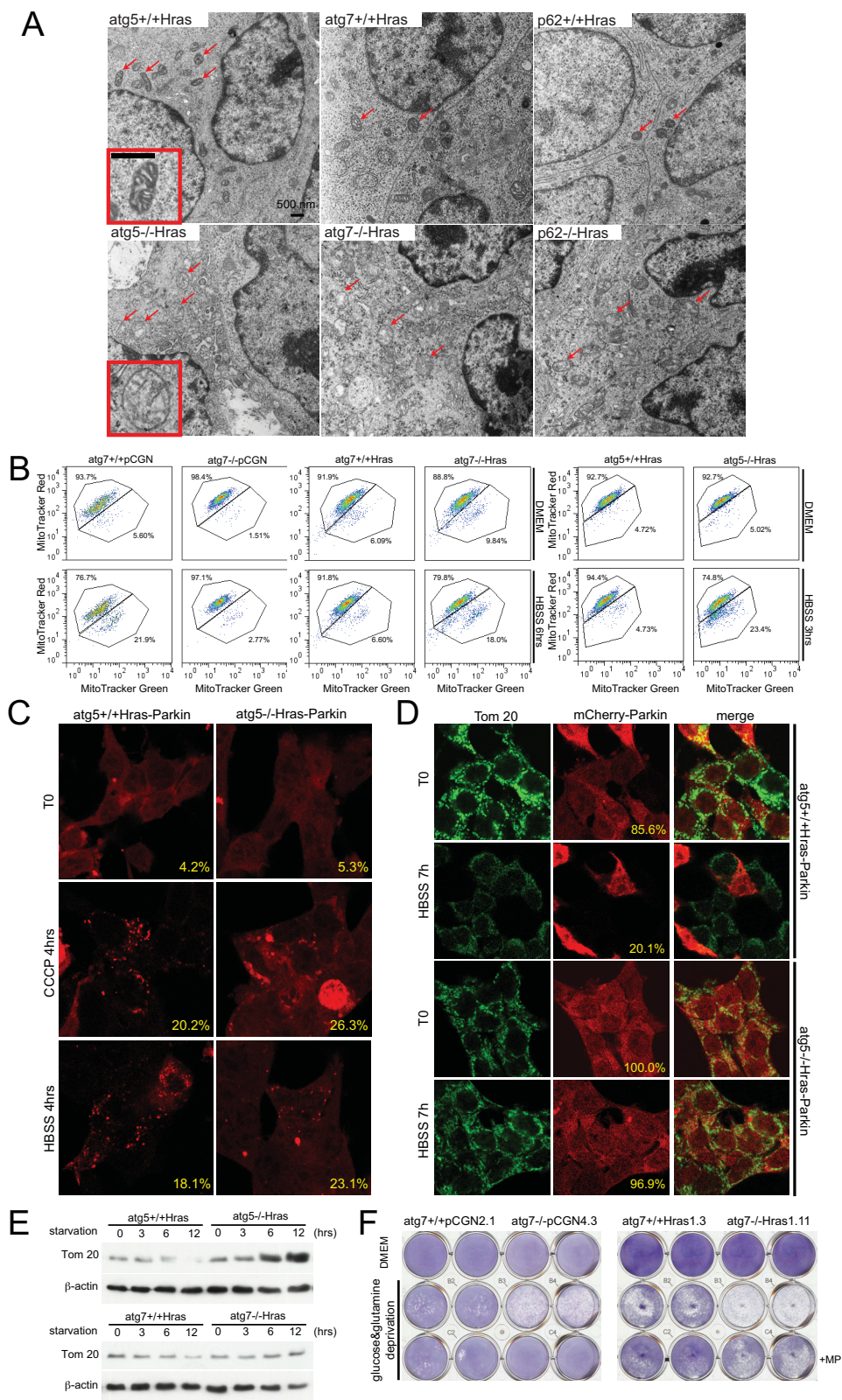


Figure 3.5 Autophagy is required to maintain mitochondrial functionality for Ras-expressing cells to survive stress

**Figure 3.6. Mitochondrial energy generation is critical for Ras-expressing cells to survive starvation.**

- (A) Diminishing functional mitochondria sensitizes H-ras<sup>V12</sup>-expressing cells to starvation. *atg7*<sup>+/+</sup>Hras and *atg7*<sup>-/-</sup>Hras cells were pre-treated with 20 mM CCCP for 1 hour and cultured in normal media or starvation for 4.5 hours in the absence or presence of CCCP. Cells were then allowed to recovery for 1 day, fixed and stained with Giemsa for clonogenic assay.
- (B) Autophagy is required for H-ras<sup>V12</sup>-expressing cells to maintain energy charge in starvation. Energy charge ( $[\text{ATP}] + [0.5\text{ADP}] / ([\text{ATP}] + [\text{ADP}] + [\text{AMP}])$ ) in control and Ras-expressing *atg5*<sup>+/+</sup> and *atg5*<sup>-/-</sup> cells under nutrient replete (DMEM) and starvation (HBSS) conditions (4 hours) is shown.

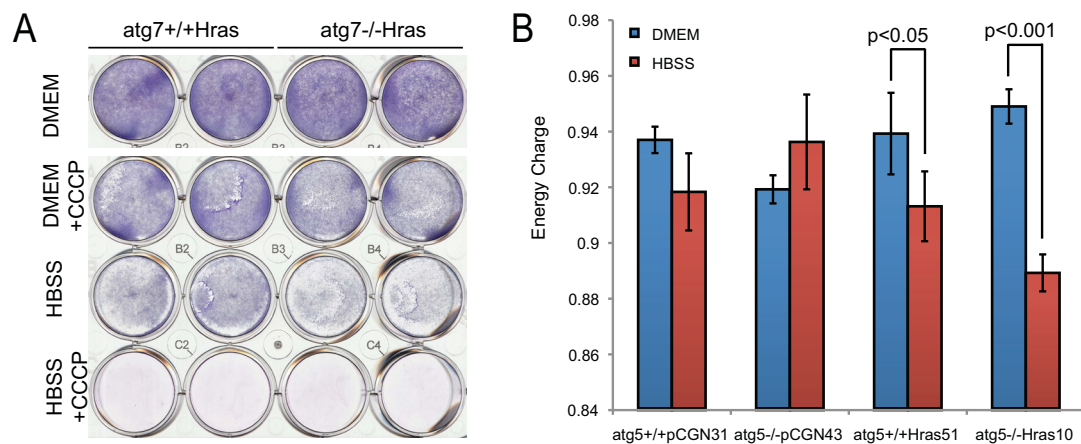


Figure 3.6 Mitochondrial energy generation is critical for Ras-expressing cells to survive starvation



## DISCUSSION

Autophagy deficiency in mice produces tissues with swollen mitochondria associated with ATP depletion, but also failure of protein and lipid homeostasis, and it is not often clear which contributes to disease manifestation (Hara et al., 2006; Komatsu et al., 2006; Komatsu et al., 2007; Komatsu et al., 2005; Kuma et al., 2004; Mathew et al., 2009; Wu et al., 2009). In post-mitotic tissues, the accumulation of autophagy protein substrates may be particularly toxic, resulting in tissue damage and disease. In contrast, tumors can lessen damaged protein accumulation through cell division, whereas damaged mitochondria may be more deleterious due to the high metabolic demand of proliferation in a stressed microenvironment. Importantly, functional mitochondria are required for Ras-transformed tumor cells to sustain viability in starvation (Figure 3.6A) and *in vivo* (Weinberg et al., 2010), this suggests that mitochondrial quality control through mitophagy is critical to maintain oxidative metabolism, energy homeostasis and viability when nutrients are limiting. Thus autophagy suppresses cancer initiation while enabling growth of aggressive cancers. In the former, autophagy prevents tissue damage that can promote cancer initiation and progression of early stage cancers. In the latter, autophagy maintains mitochondrial metabolic function important for growth of aggressive cancers. In this respect, Ras-driven tumors can be viewed as having “autophagy addiction”. Whether other oncogenic mutations act similarly, remains to be investigated.

Tumors have different levels of mitochondrial bioenergetic function (Wu et al., 2007), attributable to oncogenic mutations and activation of signaling pathways that control metabolism (Vander Heiden et al., 2009) or mutations in TCA cycle enzymes (Gottlieb and Tomlinson, 2005) that promote aerobic glycolysis (Warburg effect) (Warburg, 1956).

As some oncogenic events suppress autophagy (White and Dipaola, 2009), direct mitochondrial dysfunction derived from substrate limitation and defective mitophagy may also contribute to the Warburg effect. In the former, genetic reprogramming of metabolic pathways by mutation promotes aerobic glycolysis necessary for generation of anabolic precursors for biosynthetic pathways required for generation of new cells (Vander Heiden et al., 2009). In the latter, failure of mitochondrial quality control may prevent tumor cells from relying on oxidative metabolism to maintain cellular bioenergetics. By necessity, this may increase or select for reliance on glycolysis to support metabolism as Warburg originally described.

Cancers, such as pancreatic and colon, with a high prevalence of activating mutations in Ras, have the unfortunate distinction of a particularly poor prognosis. Our findings suggest that these cancers are most likely to respond to inhibition of autophagy, mitophagy or mitochondrial metabolism. Indeed, autophagy is elevated in these tumors that display sensitivity to autophagy inhibition (Yang et al., 2010b), consistent with the autophagy addiction model. Agents that interfere with lysosome function at the terminal step of autophagic degradation, have shown efficacy in preclinical models (Amaravadi et al., 2007; Degtyarev et al., 2008; Maclean et al., 2008) and have entered the clinic to test if autophagy inhibition can improve cancer treatment efficacy (White and Dipaola, 2009). These data now inform and support a more individualized approach to assess for activating mutations in Ras in clinical trials of autophagy inhibitors. Moreover, tumors with defective mitochondria may be particularly sensitized to agents that interfere with glucose utilization while being resistant to treatments that rely on toxic mitochondrial ROS production for cell killing.

## **Chapter 4. Targeting mTOR- and Autophagy-mediated Survival in Renal Cancer**

### **SUMMARY**

The catabolic cellular self-digestion process of autophagy is induced by metabolic stress and can promote tumor cell survival by maintaining energy homeostasis through intracellular recycling and by damage mitigation through the elimination of proteins and organelles. Autophagy is suppressed by activation of the phosphoinositol-3 kinase pathway and mammalian target of rapamycin (mTOR) that links nutrient and growth factor availability to cell growth and catabolism. mTOR inhibitors have shown efficacy in the treatment of renal cell carcinoma (RCC) but the role of autophagy stimulation and potentially counterproductive promotion of tumor cell stress tolerance was not known. We show here that the allosteric mTOR inhibitor CCI-779 (temsirolimus) promoted autophagy-mediated stress tolerance and that inhibition of autophagic degradation with the lysosomotropic agent chloroquine (CQ) enhanced kidney tumor cell death in vitro and in vivo. Further, we provide evidence that the mechanism for autophagic protection is by providing an alternative energy source and suppressing elevation of reactive species (ROS) levels. Thus, autophagy inhibition may be a useful strategy to enhance the anti-tumor activity of mTOR inhibitors in RCC.

## INTRODUCTION

Macroautophagy (autophagy) is a process whereby cellular components such as proteins, protein aggregates and organelles are captured by the formation of autophagic vesicles that then fuse to the lysosome and are ultimately degraded (Levine and Kroemer, 2008). Autophagy is activated by starvation, enabling cellular and mammalian viability by providing an internal source of building blocks for macromolecular synthesis that sustains biosynthetic and energy requirements during interruptions in nutrient availability (Kuma et al., 2004; Tsukamoto et al., 2008). Cellular stress also activates autophagy, which promotes cell survival by preventing the accumulation of damaged proteins and organelles that are a source of oxidative stress (Komatsu et al., 2007; Mathew et al., 2009). These cellular repurposing and garbage disposal roles of autophagy provide cells with the inherent flexibility to cope with environmental challenges while limiting deleterious consequences of oxidative stress. These very properties of autophagy are important to support the function of normal cells, but are also utilized by tumor cells and may be therapeutically counterproductive (Jin and White, 2008; Mathew et al., 2007a).

Metabolic stress due to oxygen, nutrient and growth factor deprivation is common in tumors due to insufficient vascularization and autophagy localizes to these hypoxic tumor regions where it supports tumor cell survival (Degenhardt et al., 2006; Karantza-Wadsworth et al., 2007; Mathew et al., 2007b). Tumor cells residing in hypoxic regions are more resistant to treatment and this autophagy conferred survival advantage has led to the realization that autophagy inhibitors may be a useful approach to improve cancer therapy (White and Dipaola, 2009). Moreover, cytotoxic and targeted cancer therapeutics induce autophagy, either due to infliction of stress and cellular

damage or due to inhibition of signaling pathways mimicking deprivation (White and Dipaola, 2009). The potential of the environmental and therapeutic autophagy induction to limit treatment effectiveness needs to be addressed.

Cells regulate growth and metabolism in response to their environment in large part through the phosphoinositide 3-kinase (PI3K) pathway and activation of the serine-threonine kinase mTOR. mTOR promotes protein synthesis, cell division and glycolysis in response to nutrient and growth factor availability, while suppressing autophagy (Guertin and Sabatini, 2007). In nutrient and growth factor replete conditions, the PI3K pathway activates mTOR through the mTORC1 complex that binds, phosphorylates and inhibits key components of the autophagy machinery that initiate autophagosome formation: the mammalian homologue of Atg13, the mammalian Atg1 homologues ULK1 and ULK2, and the focal adhesion kinase family interacting protein FIP200 (Ganley et al., 2009; Hara et al., 2008; Hosokawa et al., 2009; Jung et al., 2009). Tumor cells commonly take advantage of the cell growth-promoting function of the PI3K pathway by acquiring mutations that result in its constitutive activation (Guertin and Sabatini, 2007). As such, inhibitors of the PI3K pathway are potentially useful for restricting tumor growth. It is unclear, however, if induction of autophagy is a mechanism of resistance by increasing tumor cell stress tolerance.

mTOR inhibitors include both allosteric (rapamycin/sirolimus and other rapalogs such as temsirolimus and everolimus) and ATP-competitive mechanistic classes. Both temsirolimus and everolimus have shown efficacy in the treatment of renal cancer and are approved by the Food and Drug Administration (FDA) to treat this disease (Hudes, 2009; Motzer et al., 2008). In the first line setting, temsirolimus improves overall survival in

patients with poor prognosis, metastatic renal cell carcinoma (RCC). Unfortunately, all patients eventually relapse, contributing to the generally dismal survival statistics of this devastating disease. It is expected that most targeted therapeutics, including temsirolimus, will require combination therapy for improved therapeutic outcome. The remaining challenge is to identify the optimal approaches to enhance treatment efficacy.

If autophagy induction by mTOR inhibitors or other cancer therapeutics promotes tumor cell survival, then combination treatment with an autophagy inhibitor may be expected to promote tumor cell death. One tractable, small molecule approach to autophagy inhibition is the lysosomotropic anti-malaria drug chloroquine (CQ) and its analogues. CQ prevents lysosome acidification and thereby the degradation of the products of autophagy, resulting in autophagolysosome accumulation and persistence. In a mouse model of c-Myc-driven lymphoma, a CQ analogue hydroxychloroquine (HCQ) promoted tumor cell death by either p53 activation or alkylating agents (Amaravadi et al., 2007). In mouse models for ataxia telangiectasia and Burkitt lymphoma, CQ suppressed spontaneous tumorigenesis (Maclean et al., 2008). CQ also promoted tumor cell death in combination with the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) in a mouse model and in human samples of CML (Carew et al., 2007). While not definitive, these preclinical studies support the approach of autophagy inhibition in cancer therapy (White and Dipaola, 2009). Preclinical assessment of this approach in solid tumors has yet to be addressed.

Since the mTOR inhibitor temsirolimus (Torisel or CCI-779) has efficacy in RCC (Hudes, 2009) and promotes autophagy (Yazbeck et al., 2008) (Ravikumar et al., 2004), we assessed the functional consequences of coordinate autophagy inhibition with CQ to

the viability of kidney tumor cell lines and tumors. We found that mTOR inhibition suppressed cell proliferation and that addition of CQ promoted cell death of both apoptosis-competent or -defective mouse kidney tumor cell lines and human RCC cell lines. In combination with CCI-779, CQ promotes cell death by necroptosis and necrosis in apoptosis-defective cells, identifying a role for a RIP1-dependent cell death mechanism in response to chemotherapy. mTOR inhibition also promoted survival in metabolic stress that was partly autophagy-dependent. CQ blocked autophagic flux in tumors in vivo and enhanced the anti-tumor activity of mTOR inhibition by increasing tumor cell death. Finally, we demonstrated that supplement of respiratory substrates and attenuation of ROS is the mechanism of autophagic protection in this system. Taken together, these findings indicate that autophagy inhibition dramatically improves the efficacy of mTOR inhibitors in RCC, suggesting that combination therapy should be considered in this setting.

## RESULTS

### **mTOR inhibition is cytostatic in apoptosis-competent immortal mouse kidney cells**

To address the consequences of mTOR inhibition in cancer cells, we first examined the effects of the mTOR inhibitor temsirolimus (CCI-779), which has shown efficacy in human RCC, in the apoptosis-competent immortal mouse kidney (iBMK) cell line W2. W2 cells are non-tumorigenic due to an intact apoptotic pathway and are functional for autophagy (Degenhardt et al., 2002a; Degenhardt et al., 2006). CCI-779-treated cells were monitored with multifield time-lapse microscopy. By 24 hours, cell proliferation was suppressed in CCI-779-treated cells (untreated cells increased 290% in cell number compared to an 80% in CCI-779-treated cells) (Figure 4.1A). The cytostatic effects of CCI-779 were not accompanied by morphological indications of cell death (Figure 4.1A) or caspase-3 activation (Figure 4.1C), which would have been indicative of apoptosis at this CCI-779 concentration that is sufficient to block mTOR kinase substrate (S6) phosphorylation (see below).



**Figure 4.1. CQ in combination with mTOR inhibition promotes cell death in apoptosis-competent immortalized baby mouse kidney epithelial cells.**

- (A) Representative images from multifield time-lapse microscopy of apoptosis-competent iBMK cell line W2 under normal growth conditions and in the presence of the mTOR inhibitor CCI-779, CQ or in combination. CCI-779 suppresses cell proliferation (% increase in cell numbers is indicated) and CQ promotes cytotoxicity of CCI-779.
- (B) Viability assessment of (A) at 48 hours showing increased cell death with the combination of CCI-779 and CQ.
- (C) Western blot showing promotion of caspase-3 activation in CCI-779 treated cells by CQ.

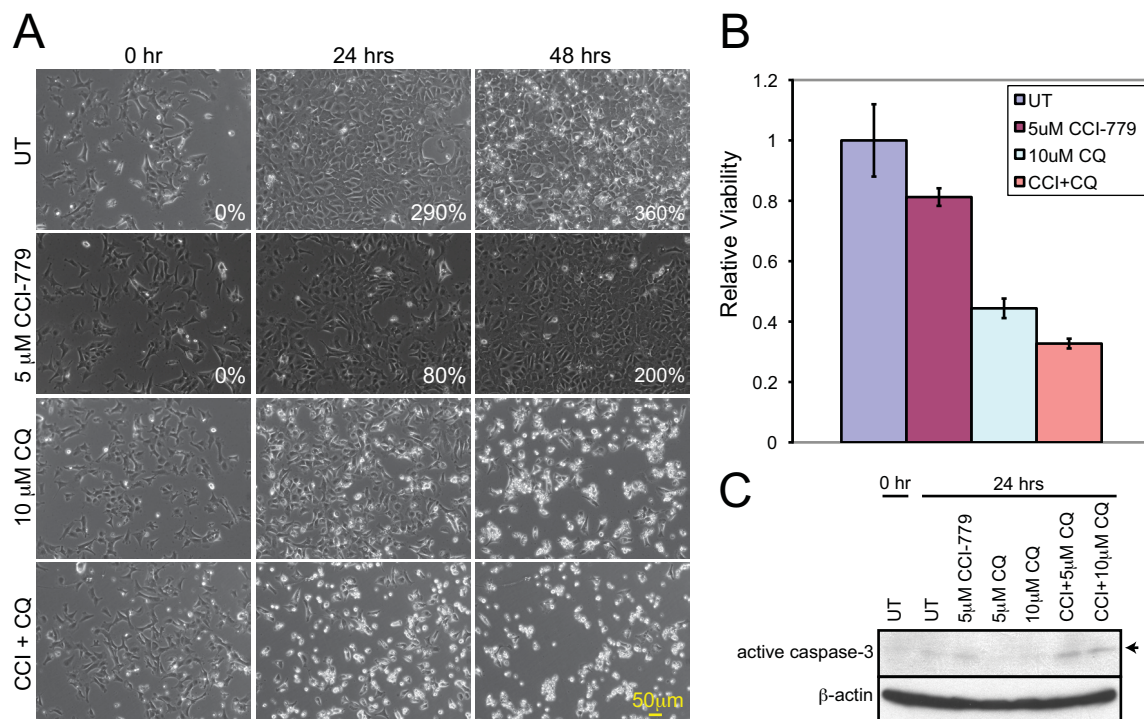


Figure 4.1 CQ in combination with mTOR inhibition promotes cell death in apoptosis-competent immortalized baby mouse kidney epithelial cells

### **The lysosomotropic agent CQ promotes cytotoxicity in combination with mTOR inhibition**

The lysosomotropic agent CQ, which blocks flux through the autophagy pathway, gradually reduced the viability of W2 cells and greatly promoted cytotoxicity in combination with CCI-779 (Figures 4.1A and 4.1B). The combination of CCI-779 and CQ promoted apoptosis as shown by caspase-3 activation (Figure 4.1C).

To further assess the functional consequences of CCI-779 and CQ, we next examined the effects of CCI-779 and CQ on apoptosis-defective Bax- and Bak- double deficient D3 cells. D3 cells are isogenic to W2 cells, but derived from a *bax*<sup>-/-</sup> *bak*<sup>-/-</sup> mouse and thereby missing the core mitochondrial apoptotic machinery rendering them apoptosis largely defective and tumorigenic (Degenhardt et al., 2002a). Due to defective apoptosis, D3 cells undergo sustained stress-induced autophagy that supports cellular survival in vitro and in tumors in vivo (Degenhardt et al., 2006; Mathew et al., 2009; Mathew et al., 2007b). Thus, use of D3 cells stably expressing the autophagy marker EGFP-LC3 greatly facilitates assessment of the functional consequences of autophagy modulation by CQ and CCI-779. CQ promoted processing of the autophagosome component LC3-I to LC3-II (Figure 4.2A) and cytosolic to membrane LC3 translocation and accumulation on autophagosome membranes as indicated by a transition from a diffuse to a perinuclear punctate LC3 localization pattern (Figure 4.2C). CQ also promoted accumulation of the autophagy substrate p62, consistent with blockage of flux through the autophagy pathway (Figure 4.2A). CCI-779 efficiently eliminated phosphorylation of the mTOR substrate S6 indicating that mTOR kinase activity was efficiently inhibited, which also occurred in untreated cells upon growth to high density (Figure 4.2B). CCI-779 increased conversion

of LC3-I to LC3-II and autophagosome formation consistent with stimulation of autophagy (Figures 4.2A and 4.2C). Furthermore, the presence of bafilomycin A1, which blocks the fusion of autophagosomes with lysosomes, further enhanced autophagosome accumulation, consistent with CCI-779 stimulating autophagy (Figure 4.2C). Multifield time-lapse microscopy demonstrated that as with apoptosis-competent W2 cells, CCI-779 suppressed cell proliferation (Figure 4.2D). CQ alone was mildly cytotoxic, and cytotoxicity indicated by loss of viability and morphological features of necrosis (cell lysis (Jin et al., 2007)) was augmented with CCI-779 (Figures 4.2D and 4.2E). Similarly, inhibition of autophagy in the presence of metabolic stress in apoptosis-defective cells resulted in cell death by necrosis (Degenhardt et al., 2006). Thus CQ enhanced cell death induction by CCI-779 was apoptosis independent.

**Figure 4.2. CQ in combination with mTOR inhibition promotes cell death in apoptosis-defective, *bax*<sup>-/-</sup>/*bak*<sup>-/-</sup> immortalized baby mouse kidney epithelial cells.**

- (A) Western blot showing LC3-II and p62 accumulation and turnover upon 12 hours treatment of the apoptosis-defective *bax*<sup>-/-</sup>/*bak*<sup>-/-</sup> iBMK cell line D3 stably expressing EGFP-LC3 (D3-EGFP-LC3) with CQ and CCI-779 respectively.
- (B) Western blot time course showing suppression of S6 phosphorylation by CCI-779 in D3 cells.
- (C) CQ blocked and CCI-779 stimulated flux through the autophagy pathway. D3-EGFP-LC3 cells were treated with CQ for 1 day and assessed for punctate localization of LC3, which indicates autophagosome formation. CQ induced remarkable accumulation of autophagosomes consistent with blockage of autophagosome degradation in lysosomes. CCI-779 induced autophagosome formation and turnover, and inhibition of lysosome function with bafilomycin A1 promoted autophagosome accumulation in CCI-779-treated cells indicative of autophagy induction with CCI-779. Percentage of cells with LC3 puncta is indicated.
- (D) Representative images from multifield time-lapse microscopy of D3 cells under normal growth conditions and in the presence of CCI-779 without and with CQ. CCI-779 suppressed cell proliferation (% increase in cell numbers is indicated) and CQ promoted cytotoxicity of CCI-779-treated cells.
- (E) Viability assessment of (A) at 48 hours showing increased cell death with the combination of CCI-779 and CQ.

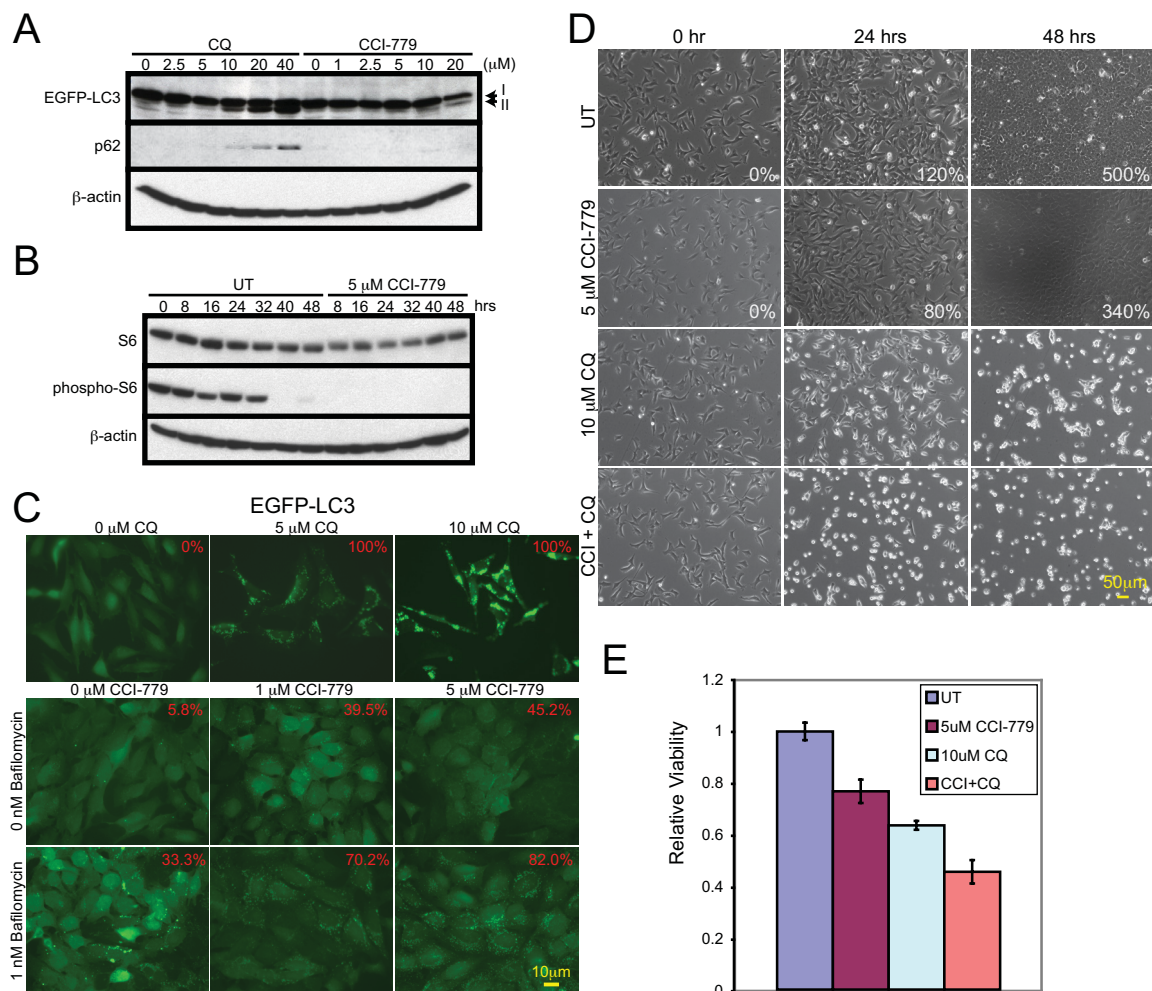


Figure 4.2 CQ in combination with mTOR inhibition promotes cell death in apoptosis-defective, *bax*<sup>-/-</sup>/*bak*<sup>-/-</sup> immortalized baby mouse kidney epithelial cells

**CCI-779 and CQ induce cell death by necrosis and necroptosis**

Cell death, particularly of apoptosis-defective cells can occur by necrosis or a programmed form of necrosis, necroptosis, which is signaled through death receptors and RIP1 and RIP3 kinases (Galluzzi and Kroemer, 2008). To assess the type of necrosis induced by combination of CQ and CCI-779, the apoptosis-defective D3 cells were treated with CCI-779, CQ separately and in combination, in the absence and presence of the RIP1 kinase and necroptosis inhibitor necrostatin-1 (Degterev et al., 2008). Necrostatin-1 alone or with CCI-779 or CQ did not significantly alter D3 cell viability; however, Necrostatin-1 increased viability in the presence of both CCI-779 and CQ (Figures 4.3A and 4.3B). Necrostatin-1 also increased clonogenic survival of CCI-779 and CQ combination-treated cells, indicating that inhibition of necroptosis protected cells sufficiently for a durable recovery from CCI-779 and CQ (Figure 4.3C). Since Necrostatin-1 did not completely protect from CCI-779- and CQ-induced cell death, this suggests that necroptosis and necrosis both contribute to cell death of apoptosis-defective kidney tumor cells.

**Figure 4.3. CQ enhances cytotoxicity of CCI-779 by inducing necroptosis.**

- (A) Viability assessment of D3 cells with CCI-779 or CQ alone or in combination, in the absence and presence 30  $\mu$ M of Necrostatin-1 for 78 hours. Necrostatin-1 increased viability in CCI-779 and CQ combination-treated cells. The number represents the fold of viability of CCI-779 and CQ combination-treated cells in the absence and presence of Necrostatin-1.
- (B) Representative images showing Necrostatin-1-mediated protection on CCI-779 and CQ combination-treated cells.
- (C) Clonogenic survival of D3 cells in normal growth and upon treatment with CCI-779 and CQ in combination without or with Necrostatin-1 showing increase of viable cells with proliferation capability in the presence of Necrostatin-1 in combination-treated cells. Cells were left untreated or were treated with CCI-779 and CQ in combination without or with Necrostatin-1 for 4 days and then allowed to recover without drugs for 3 days followed by Geimsa staining.



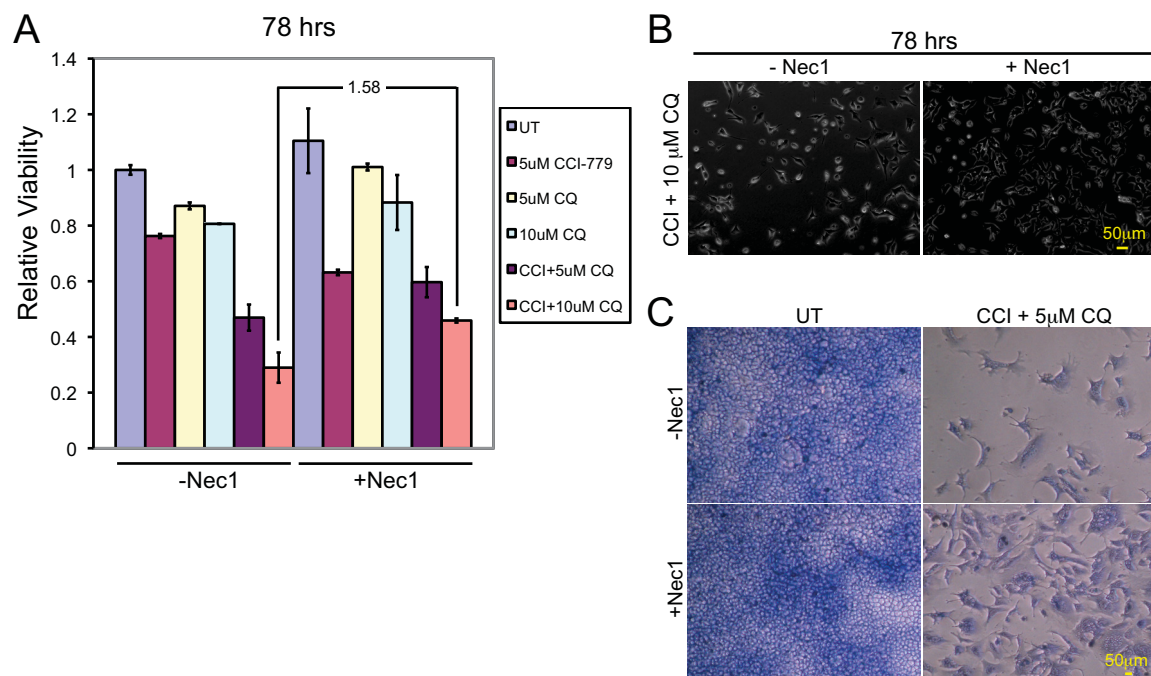


Figure 4.3 CQ enhances cytotoxicity of CCI-779 by inducing necroptosis

## **mTOR inhibition provides protection from metabolic stress that is antagonized by CQ**

Since metabolic stress is common in tumors due to insufficient vascularization and autophagy is induced and supports cell survival in these hypoxic regions, we next sought to investigate the consequence of mTOR inhibition and autophagy modulation when tumor cells are under metabolic stress. It was possible that CCI-779, by inducing autophagy, provided protection from metabolic stress that could be reversed with CQ.

Autophagy was induced in D3 cells by metabolic stress without and with CCI-779 as indicated, and monitored by EGFP-LC3-marked autophagosome formation and turnover as LC3 is degraded via autophagic flux (Figures 4.4A and 4.4B). CQ caused the accumulation of autophagosomes under metabolic stress in the absence and presence of CCI-779 (Figure 4.4A) and suppressed the turnover of LC3-II and p62 (Figure 4.4B). Reduced accumulation of LC3-II and p62 in CQ-treated cells in the presence of CCI-779 suggested that CCI-779-stimulated autophagic flux might not be completely blocked by CQ (Figure 4.4B). After 5 days of metabolic stress, D3 cells displayed markers of autophagy but also endoplasmic reticulum and oxidative stress along with decreased cytoskeletal components that accompany striking changes in morphology, yet remain viable (Mathew et al., 2009) (Figures 4.4C and 4.4D). mTOR inhibition with CCI-779 markedly suppressed these stress-induced morphological alterations, and this protection was partly reversed by CQ (Figure 4.4C). While it was not possible to assess a survival advantage provided by CCI-779 in metabolic stress in Bax/Bak-deficient apoptosis-defective D3 cells, CCI-779 promoted cell survival in apoptosis-competent W2 cells (Figure 4.4E), which are susceptible to apoptotic cell death in metabolic stress

(Degenhardt et al., 2006). Furthermore, CQ nicely reversed the survival advantage in metabolic stress provided by CCI-779 and cooperated with CCI-779 to induce cell death in apoptosis-competent W2 cells (Figure 4.4E). This suggests that inhibiting mTOR may have an undesirable property of providing tumor cells with a survival advantage in response to metabolic stress that CQ may be useful to counteract.

**Figure 4.4. CCI-779 provides protection from metabolic stress that is suppressed by CQ.**

- (A) Autophagy induction by metabolic stress and CCI-779 and inhibition of autophagic flux by CQ. D3-EGFP-LC3 cells were subjected to metabolic stress for 48 hours in the absence and presence of CCI-779, CQ alone or in combination. Numbers indicate the percentage of cells with punctate EGFP-LC3 localization. Metabolic stress induced autophagy and CCI-779 promoted LC3 punctation. CQ induced striking accumulation of autophagosomes indicative of blockage of autophagosome degradation.
- (B) Western blot of time course showing LC3 I/II conversion and turnover and p62 accumulation under metabolic stress conditions and in the presence of CCI-779 or CQ separately or in combination.
- (C) Representative images of D3 cells after 5 days of metabolic stress without or with CCI-779, CQ or in combination showing protection from metabolic stress morphologically by CCI-779 and suppression of the CCI-779 provided protection by CQ.
- (D) Viability assessment of (C).
- (E) Viability assessment of apoptosis-competent W2 cells under metabolic stress and treated with CCI-779 or CQ alone or in combination for 3 days showing protection from metabolic stress by CCI-779 and suppression of the protection by CQ. Moreover, CQ cooperated with CCI-779 to cause loss of viability.

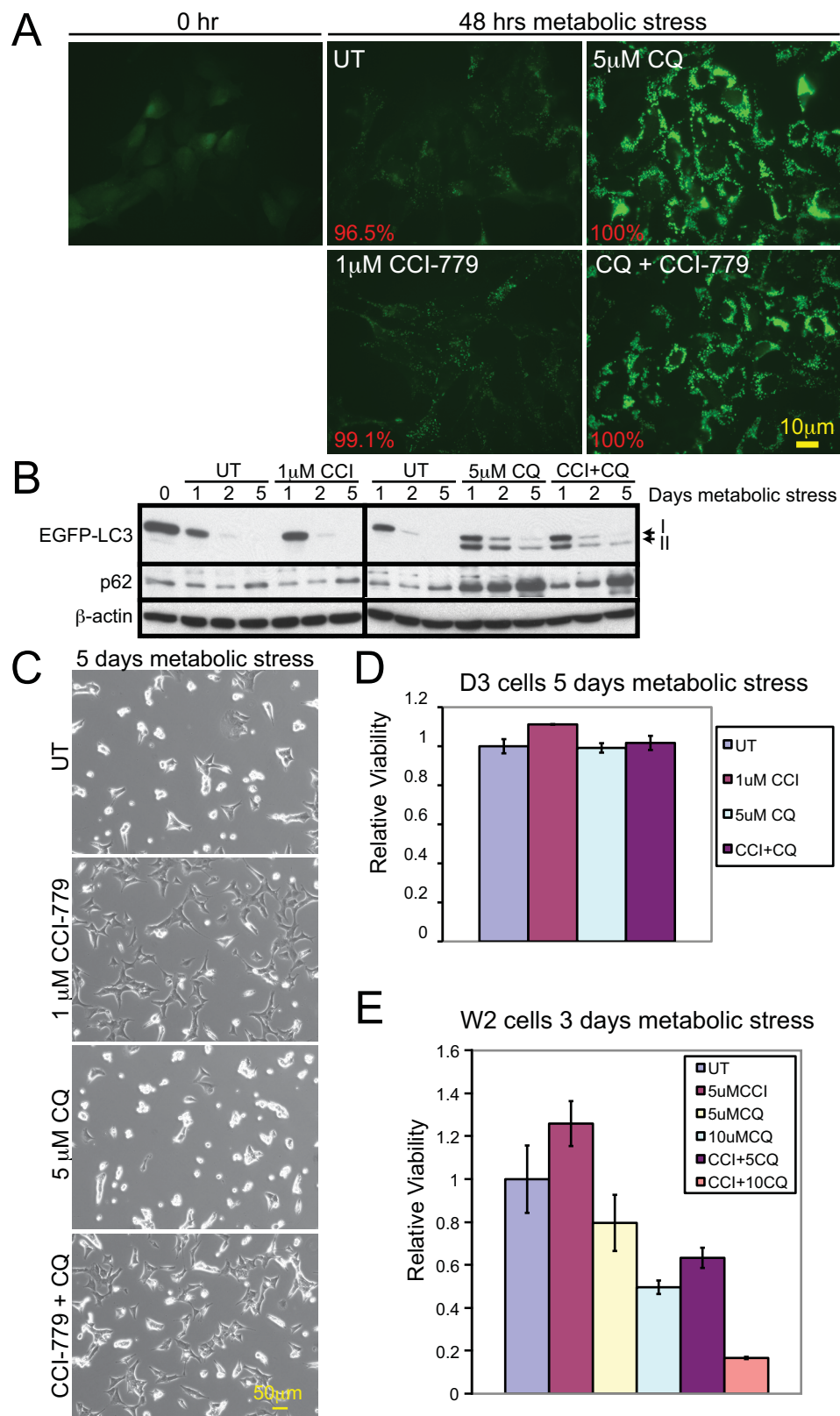


Figure 4.4 CCI-779 provides protection from metabolic stress that is suppressed by CQ

### **Protection from metabolic stress by mTOR inhibition is partly autophagy-dependent**

Since CCI-779 provided a survival advantage in response to metabolic stress and also stimulated autophagy, we next sought to determine if this enhanced survival was autophagy-dependent. To test this hypothesis, we generated iBMK cell lines (Figure 4.5A) from *atg7<sup>+/+</sup>* and autophagy-defective *atg7<sup>-/-</sup>* mice (Komatsu et al., 2005). *Atg7<sup>+/+</sup>* and *atg7<sup>-/-</sup>* iBMK cell lines were engineered to stably express Bcl-2 to suppress apoptosis in order to better assess autophagy function (Figure 4.5B). *Atg7<sup>-/-</sup>* iBMK cell lines were confirmed to possess a profound defect in autophagy compared to *atg7<sup>+/+</sup>* cells, indicated by impairment of autophagosome formation under metabolic stress or with CQ (Figure 4.5C). These autophagy-competent and -defective iBMK cell lines were subjected to metabolic stress and treated with CCI-779 or CQ alone and in combination. As reported previously for autophagy-defective *beclin1<sup>+/-</sup>* and *atg5<sup>-/-</sup>* iBMK and iMMEC cell lines (Degenhardt et al., 2006; Karantza-Wadsworth et al., 2007; Mathew et al., 2009; Mathew et al., 2007b), deficiency in *atg7* conferred susceptibility to metabolic stress (Figures 4.5D and 4.5E). CCI-779 provided dramatic protection of Bcl-2-expressing, autophagy-competent *atg7<sup>+/+</sup>* cells, while the protection of autophagy-defective cells was significantly reduced, indicating that the protection is partly autophagy-dependent (Figures 4.5D and 4.5E). The increased protection of Bcl-2-expressing, compared to Bax/Bak-deficient iBMK cells by CCI-779 is likely due to the less efficient apoptotic block provided by Bcl-2 (Figures 4.4D and 4.5E). CQ reduced cell survival to a lesser extent in autophagy-defective cells, implying that its cytotoxicity under metabolic stress is partly through blockage of autophagy. Importantly, addition of CQ antagonized the

CCI-779 protection more so in *atg7* wild type compared to *atg7*-deficient cells (30.0% and 50.3% reduced viability of wild type compared to 25.3% and 10.2% reduced viability of *atg7*-deficient cells) (Figure 4.5E).

**Figure 4.5. Protection from metabolic stress by CCI-779 is partly autophagy-dependent.**

(A) Generation of immortal *atg7<sup>+/+</sup>* and *atg7<sup>-/-</sup>* baby mouse kidney epithelial cell lines.

Western blot in multiple cell lines showing expression of the epithelial cell marker  $\beta$ -catenin. Expression of E1A and dominant-negative p53 that target the Rb and p53 pathways respectively to facilitate cell immortalization is also shown.

(B) Generation of ATG7 cell lines expressing Bcl-2. Human Bcl-2 was transfected into *atg7<sup>+/+</sup>* and *atg7<sup>-/-</sup>* cell lines. Genotyping of cell lines: 3kb bands indicate *atg7<sup>+/+</sup>* and 2kb bands indicate *atg7<sup>-/-</sup>* (Top panel). Western blot showing comparable expression of Bcl-2 (Bottom panel).

(C) Functional characterization of ATG7 cell lines. *atg7<sup>+/+</sup>* and *atg7<sup>-/-</sup>* cells were transfected with EGFP-LC3 (cell line numbers shown in top left corners). One day post-transfection, cells were subjected to metabolic stress for 12 hours and assessed for induction of autophagy. Autophagy was induced in wild type but not in *atg7<sup>-/-</sup>* cell lines as indicated by cells with punctate EGFP-LC3 localization (% shown in bottom left corners) (Top panel). EGFP-LC3-transfected Bcl-2-expressing ATG7 cells were treated with CQ for 16 hours and autophagosome formation was assessed. Numbers indicate the percentage of cells with LC3 translocation. Bcl-2-expressing *atg7<sup>-/-</sup>* cells failed to form autophagosomes (Bottom panel).

(D) Representative images showing protection from metabolic stress of autophagy-competent *atg7<sup>+/+</sup>* cells by CCI-779 and reduced protection of autophagy-deficient *atg7<sup>-/-</sup>* cells.



(E) Viability assessment of (D) and an additional cell line in each genotype showing less protection by CCI-779 in Bcl-2-expressing *atg7<sup>-/-</sup>* cells compared to *atg7<sup>+/+</sup>* cells under metabolic stress. The CQ-mediated suppression of CCI-779-provided viability protection was less in autophagy-defective Bcl-2-expressing *atg7<sup>-/-</sup>* cells than in Bcl-2-expressing *atg7<sup>+/+</sup>* cells. Numbers represent the relative decrease in cell viability caused by CQ in the presence of CCI-779.

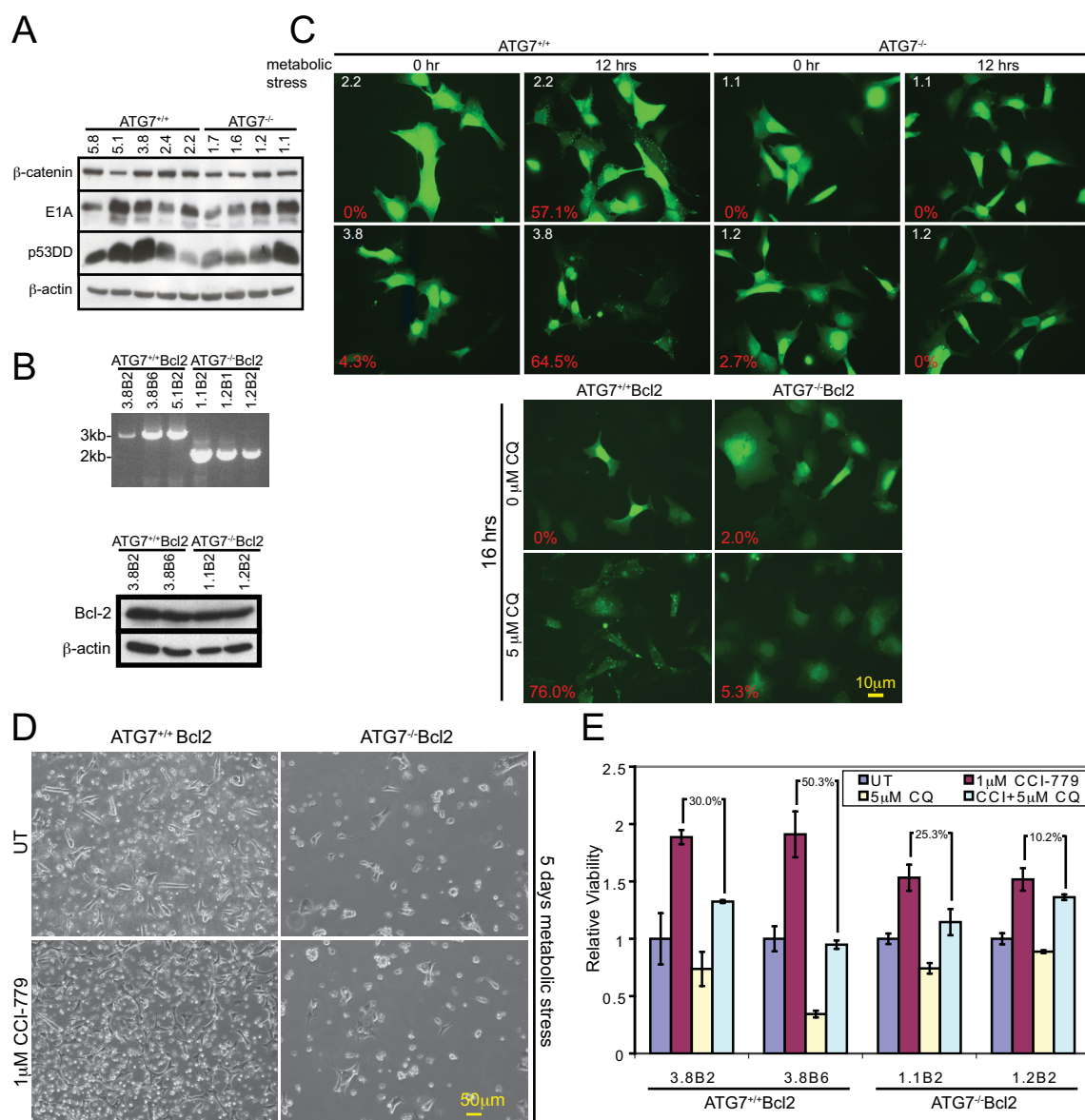


Figure 4.5 Protection from metabolic stress by CCI-779 is partly autophagy-dependent

### **CQ induces autophagosome formation in tumors and enhances the anti-tumor activity of CCI-779**

Given that in vitro findings support blocking autophagy with the lysosomotropic agent CQ to enhance cytotoxicity of CCI-779, we examined whether this occurred in tumor allografts in vivo. D3 cells stably expressing the autophagosome marker EGFP-LC3 (Degenhardt et al., 2006) were established as tumors in nude mice then treated. CQ administration promoted autophagosome accumulation in D3 tumors indicative of autophagy blockage (Figure 4.6A). CCI-779 alone was more effective at suppressing tumor growth than CQ, and in combination with CCI-779, CQ further suppressed tumor growth (Figure 4.6B). Following 16 days of treatment, tumors were excised and examined for cell death, autophagy induction, and loss of S6 phosphorylation. CCI-779 and CQ and the combination of the two, increased EGFP-LC3 puncta indicative of autophagosome formation in tumors compared to tumors from vehicle-treated control mice (Figure 4.6C). Immunohistochemical analysis of tumors showed inhibition of mTOR (loss of phospho-S6) by CCI-779 (Figure 4.6C). In the CQ-treated mice, the phospho-S6 was also low, which may potentially reflect negative feedback suppressing mTOR activity or preferential elimination of phospho-S6-positive tumor cells by CQ. Histological analysis showed that the combination of CCI-779 and CQ strikingly increased the number of tumor cells undergoing necrotic cell death compared to single agent or vehicle-treated mice (Figures 4.6C and 4.6D). No significant increase in apoptosis, indicated by immunohistochemistry for active caspase-3, was observed in any of the tumors (data not shown) consistent with necrotic cell death mediating suppression of tumor growth. These findings support inhibition of mTOR with CCI-779 in

combination with autophagy inhibition with CQ to increase therapeutic efficacy in the treatment of kidney tumors (Figure 4.7G).

**Figure 4.6. CQ enhances anti-tumor activity in combination with mTOR inhibition.**

- (A) CQ induces autophagosome accumulation in mouse allografts. Established D3-EGFP-LC3 tumors (tumor volume about 200 mm<sup>3</sup>) were treated with CQ. One-day after CQ administration, tumors were excised and processed for frozen sections to preserve EGFP-LC3 fluorescence. Arrows indicate cells with autophagosomes. CQ induced autophagosome accumulation indicative of blockage of autophagy flux.
- (B) Anti-tumor activity of CCI-779, CQ alone or in combination in mouse allografts. Established D3-EGFP-LC3 tumors were treated with CCI-779 or CQ or in combination. Arrowheads present administration of CCI-779 and arrows present administration of CQ. CCI-779 and CQ alone had some anti-tumor activity. CQ enhanced the anti-tumor activity of CCI-779. During dosing period (day16), tumor materials were taken for histology and immunohistochemistry (indicated with a black arrow).
- (C) Tumor sections of (B) showing inhibition of phospho-S6 with CCI-779 and promotion of autophagosome formation in the presence of CCI-779, CQ alone or in combination. Tumor histology showing induction of necrosis by CQ and CCI-779 combination. Yellow arrows indicate necrotic cells. Red arrows indicate cells with punctate EGFP-LC3.
- (D) Quantification of necrotic cells in (C). The percentage of necrotic cells was determined by counting necrotic cells out of a population of 400 cells. CQ enhanced the anti-tumor activity of CCI-779 by inducing necrotic cell death.

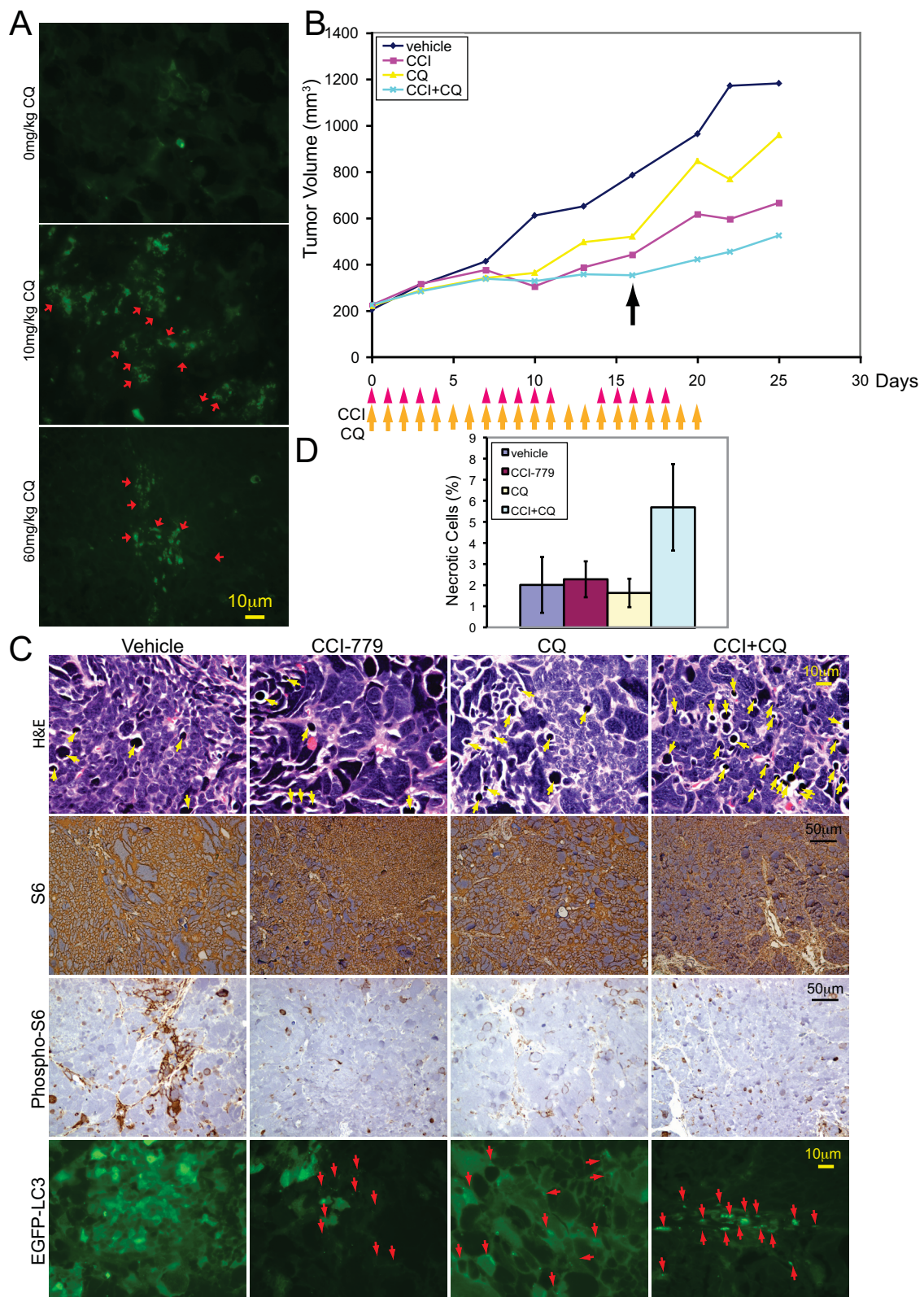


Figure 4.6 CQ enhances anti-tumor activity in combination with mTOR inhibition

**Reactive oxygen species (ROS) production and metabolic catastrophe contribute to the cytotoxicity of CCI-779 and CQ combination.**

Since inhibition of autophagy reversed the stress protection of mTOR inhibition and cooperated with mTOR inhibition to induce cell death and suppress tumor growth, we sought to address how inhibition of autophagy and mTOR concomitantly lead to cell death. In addition, because the combination of CCI-779 and CQ showed promising cytotoxic effects on mouse kidney cell lines, we sought to determine if the paradigm was also valid in human RCC cell lines. mTOR promotes glucose uptake and glycolysis in response to growth factor and nutrient availability (Shaw and Cantley, 2006; Wullschlegel et al., 2006; Pouyssegur et al., 2006). Inhibition of mTOR therefore mimics deprivation. Since induction of autophagy, which accompanies mTOR inhibition can provide alternative energy source by intracellular recycling, we hypothesized that concomitant inhibition of autophagy with mTOR would lead to metabolic catastrophe and elevated ROS levels, which eventually cause cell death. Indeed, supplement of the respiratory substrate methyl-pyruvate (MP) rescue viability and clonogenic survival of cells treated with CCI-779 and CQ combination (Figure 4.7A-4.7D). By adding the general ROS scavenger N-acetylcysteine (NAC) cell viability and clonogenic ability of cells treated by CCI-779 and CQ combination can be restored (Figure 4.7C and 4.7D). We also examined the mitochondrial superoxide and cellular ROS levels respectively using MitoSox Red fluorogenic dye, which specifically reacts with mitochondrial superoxide, and 2', 7'-dichlorofluorescein diacetate (DCF-DA), which predominantly reacts with H<sub>2</sub>O<sub>2</sub>. In consistent with the hypothesis, ROS levels increased in cells treated with CCI-779 and CQ in combination (Figure 4.7E and 4.7F).



**Figure 4.7. Concomitant inhibition of autophagy with mTOR leads to cell death that is reversed by N-acetylcysteine (NAC) or methyl-pyruvate (MP).**

- (A) Representative images of D3 cells treated with CCI-779 or CQ alone, or in combination in the absence or presence of 10 mM MP for 2 days.
- (B) Viability assessment of (A). MP reversed the CCI-779 and CQ combination induced cell death.
- (C) NAC and MP reversed the loss of viability of human renal cell carcinoma cell line (RCC4) treated with combination of CCI-779 and CQ. Cells were treated with drugs for 30 hours.
- (D) Clonogenic survival assay showing restoration of NAC or MP on clonogenic capability of CCI-779 and CQ combination treated RCC4 cells. Cells were treated with drugs for 30 hours, allowed to recover for 2 days and stained with Giemsa.
- (E) Combination of CCI-779 and CQ caused elevated mitochondrial superoxide levels in RCC4. Mitochondrial superoxide was detected with MitoSOX Red fluorogenic dye.
- (F) Combination of CCI-779 and CQ led to elevated ROS levels. ROS levels in RCC4 were detected by flow cytometry using the ROS sensor (2', 7'-dichlorofluorescein diacetate (DCF-DA)).
- (G) Model for suppression of the cytoprotective effect of autophagy on mTOR inhibition.



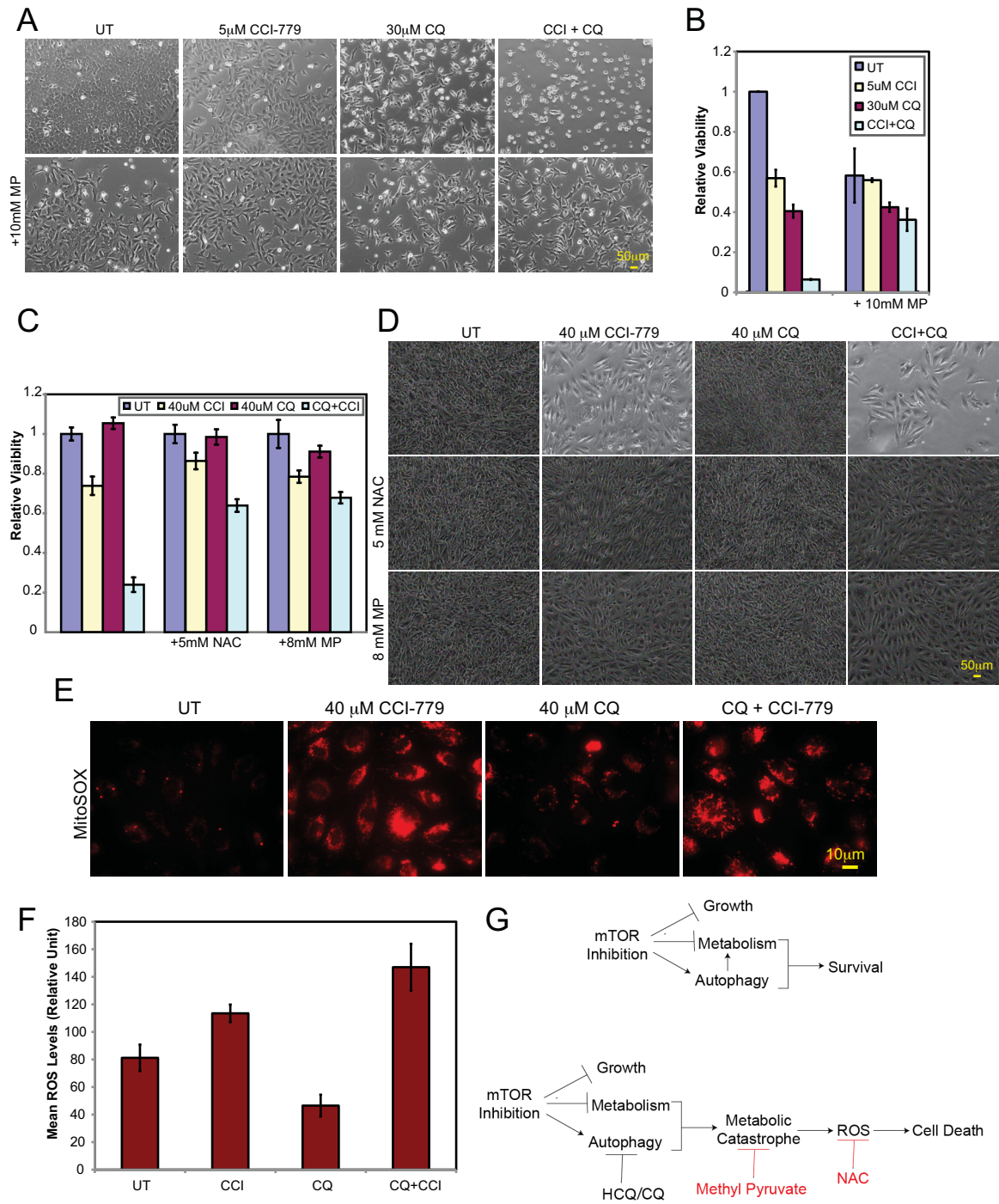


Figure 4.7 Concomitant inhibition of autophagy with mTOR leads to cell death that is reversed by N-acetylcysteine (NAC) or methyl-pyruvate (MP)

## DISCUSSION

RCC is a disease with high mortality and allosteric mTOR inhibitors (rapalogs) such as temsirolimus and everolimus have efficacy but are not curative (Hudes, 2009; Motzer et al., 2008). One obvious strategy being implemented is to combine rapalogs with inhibitors that target other pathways activated in RCC, such as the VEGF pathway to improve outcome (Merchan et al., 2009). Another is to target the mTOR negative feedback loop with ATP-competitive dual PI3K/mTOR inhibitors or AKT inhibitors, which prevent Akt activation that is counterproductive. Alternatively, if autophagy activation by mTOR inhibitors has the potential to promote tumor cell survival, then therapeutic strategies to block autophagy downstream of mTOR are warranted (White and Dipaola, 2009) (Figure 4.7G).

To establish if suppression of autophagy improves anti-cancer activity with mTOR inhibition, first it was necessary to document the functional role of autophagy activation by rapalogs such as temsirolimus. Second, if autophagy induction was survival-promoting in this setting, then determining if autophagy inhibition enhanced the anti-tumor activity of temsirolimus would be important. Indeed, suppression of autophagy by either genetic or pharmacologic means increased the cytotoxicity of temsirolimus, supporting a pro-survival role for autophagy. Furthermore, inhibition of autophagy genetically or pharmacologically with the lysosomotropic agent CQ enhanced cytotoxicity of temsirolimus in vitro and in tumors in vivo.

mTOR inhibition increased survival to metabolic stress, an activity most evident in Bcl-2-expressing rather than Bax/Bak-deficient cells, suggesting that an suppression of apoptosis may contribute to mTOR-mediated stress protection. Tumor cells residing in

hypoxic regions are more therapy resistant, and if inhibiting mTOR can provide an additional advantage, this may confound treatment effectiveness. The ability of mTOR to promote survival in metabolic stress was in part dependent on autophagy, since deficiency in *atg7* reduced viability in this setting. Other consequences of mTOR inhibition that may also enhance stress survival have not been identified, but suppression of cell growth and alteration of metabolic status of cells are likely candidates.

If so, then efficacy of mTOR inhibitors may also be improved by manipulating tumor cell metabolism.

CQ and HCQ are lysosomotropic agents that interfere with lysosome function and as such block the degradation of the products of the autophagy process (Klionsky et al., 2008). Although other activities have also been ascribed to CQ, it is clear that CQ blocks flux through the autophagy pathway and enhanced cytotoxicity of temsirolimus that was partly dependent on functional autophagy status. Although perturbation of lysosome function in cancer may have additional benefits by directly enhancing cell death (Kirkegaard and Jaattela, 2009), this suggests that autophagic degradation of cellular contents in lysosomes is important for survival of cancer cells in stress. Indeed, CQ and autophagy inhibition increases sensitivity to proteasome inhibitors including bortezomib (Velcade) in cancer cell lines (Ding et al., 2009; Mathew et al., 2009), imantinib (Gleevec) in CLL cell lines and primary patient samples (Bellodi et al., 2009), AKT inhibitors in PTEN deficient cell lines and tumors (Degtyarev et al., 2008), and to alkylating agents in glioma cell lines and xenographs (Amaravadi et al., 2007). All of these underscore the role of autophagy in clearance of damaged proteins and organelles

as well as in replenishment of metabolites or maintenance of energy status to enhance survival to stress.

Given mTOR links the growth factor as well as nutrient availability to cell growth that is intertwined with metabolism and mTOR has been shown to upregulate nutrient uptake and glycolysis (Shaw and Cantley, 2006); (Pouyssegur et al., 2006; Wullschleger et al., 2006). It would not be surprising that inhibiting mTOR, like starvation, would poise cells from utilizing glycolysis, which is preferred by proliferating cells or tumor cells, to oxidative phosphorylation (OXPHO), which is favored by cells under starvation or in quiescence (Vander Heiden et al., 2009). Mitochondria serve as the primary intracellular source of reactive oxygen species (ROS); even in otherwise tightly coupled electron transport chain, 1-3% of oxygen is not completely reduced. Shifting to OXPHO would increase the risk of ROS production. It has been reported that starvation elevates ROS levels (Ahmad et al., 2005; Scherz-Shouval et al., 2007). This may result from increase of ROS production because of regulated processes for signal transducing purposes or accumulation of malfunctioned mitochondria (Degtyarev et al., 2008; Scherz-Shouval et al., 2007) or from decrease of detoxification of ROS because of insufficient pyruvate and NADPH, which are believed supportive for detoxification of hydroperoxides (Averill-Bates and Przybytkowski, 1994; Nath et al., 1995) during starvation. Here we showed mTOR inhibition elevated ROS levels (Figure 4.7E and 4.7F). Concomitant block of autophagic degradation with mTOR inhibition exacerbated this situation and led to cell death. NAC or MP rescued cell from death in this setting, suggesting that failure to eliminate mitochondria that produce ROS and to supply mitochondrial respiratory

substrate by recycling internal resource leads to metabolic catastrophe, which in turn amplifies ROS production and cause cell death.

In contrast, in some settings excessive autophagy is associated with cell death, and interestingly, small molecules have been identified that promote this form of cell death specifically in VHL-negative RCC cell lines and xenograft tumors (Turcotte et al., 2008). Going forward it will be of great interest to establish the mechanism by which excessive autophagy stimulation may lead to cell death as well as exploiting the utility of lysosomotropic agents to block autophagy to promote cell death.

In most cases, including in mammalian development, autophagy deficiency promotes cell death, and this is usually associated with cellular stress (Levine and Kroemer, 2008). Reduced autophagy can promote apoptosis (Boya et al., 2005; Degenhardt et al., 2006), necrosis (Degenhardt et al., 2006), and now also necroptosis. Necroptosis is a specialized form of programmed necrosis initiated by death receptor signaling and mediated by RIP1 and RIP3 kinases (Declercq et al., 2009; Degterev et al., 2008). Where inhibition of autophagy under conditions of metabolic stress is expected to induce necrosis due to the inability of cells to sustain energy production and suppress oxidative stress (Jin et al., 2007; Mathew et al., 2009), how necroptosis is triggered is not known. It is interesting to speculate that the elevated stress associated with defective autophagy promotes autocrine and paracrine TNF- $\alpha$  production, which is a known activator of necroptosis. These findings also implicate necroptosis as a death pathway relevant in cancer therapy, as emerging evidence suggests (Horita et al., 2008; Hu et al., 2007). It will be important to establish if necroptosis can be exploited, as the apoptosis pathway has been (Tse et al., 2008), for the development of targeted cancer therapeutics.

Although agents such as bevacizumab, sorafenib or sunitinib, which target the VEGF pathway and inhibitors of the mTOR pathway, such as temsirolimus and everolimus, have been demonstrated to have clinical activity against advanced RCC, this benefit is only temporary. One approach to improve results includes ongoing studies to assess combinations of these targeted agents in clinical trials (Gray et al., 2006). Our findings support a newer paradigm to abrogate mTOR inhibitor resistance through inhibition of autophagy, and provide a rationale for the combination of temsirolimus and CQ in clinical trials. Testing the clinical effectiveness of interfering with autophagy in patients receiving mTOR inhibitors, and assessing the importance of inhibiting autophagy in combination with other targeted therapies, is now justified.

## Chapter 5. DISCUSSION

### Ras activation upregulates autophagy

#### *Mechanism of autophagy upregulation in Ras-expressing cells*

Human cancer cell lines that aberrantly activate Ras generally showed high basal autophagy (Guo et al., 2010). iBMK cells stably expressing H-Ras<sup>V12</sup> displayed elevated autophagy compared to the vector control cells (Figures 3.1B and C). Therefore, autophagy induction is likely a direct consequence of Ras activation rather than an outcome of selection *in vitro* or *in vivo*. The mechanism of autophagy upregulation by Ras expression is still unknown. Under normal culture conditions the energy charge of iBMK cells without and with Ras-expressing was comparable regardless of the autophagy competency (Figure 3.6B), suggesting that autophagy upregulation is not caused by Ras-induced high metabolic demand and energy depletion in normal culture conditions. In normal cultured Ras-expressing cells, the level of phosphorylated S6 protein indicative of active mTORC1 was similar to that of vector control cells (data not shown). Thus, Ras induces autophagy in an mTORC1-independent manner. Ras has been suggested stabilizing HIF1 $\alpha$ , which induces autophagy, under hypoxia (Blum et al., 2005). However, the contribution of this activity to autophagy induction is unknown. Whether other Ras-caused effects such as the high levels of metabolic waste or Ras downstream molecular pathways stimulate autophagy remains to be investigated.

#### *Physiological significance of autophagy induction by Ras expression*

Autophagy deficiency impaired tumorigenesis of Ras-expressing iBMKs (Figures 3.3A and B). This suggests that the Ras-associated autophagy contributes to the aggressive phenotype of Ras-expressing tumors. There were no apparent growth or

proliferative defects in autophagy-deficient cells under normal culture (Figures 3.1D and E), indicating that autophagy is not required for Ras-expressing cells in nutrient replete conditions. Deprivation of nutrients and growth factors (HBSS) readily killed autophagy-deficient Ras-expressing cells while those autophagy-competent still sustained for more than 16 hours, indicating that autophagy is necessary to support the viability and proliferation of Ras-expressing cells under nutrient and growth factor limited conditions. Autophagy deficiency caused sensitivity to glucose and glutamine double deprivation that was rescued by the mitochondrial substrate methyl-pyruvate (MP) (Figure 3.5F), suggesting that autophagy supplies substrates for mitochondrial metabolism. The sensitivity of autophagy-deficient cells to starvation showed some variations, suggesting that in addition to supporting mitochondrial substrates directly, autophagy may have other roles for Ras-expressing cells to survive starvation.

#### **Autophagy specifically supports tumorigenicity of Ras-expressing cells**

Autophagy deficiency impairs tumorigenicity of Ras-expressing cells while promotes tumorigenicity of Bcl-2-expressing cells (Mathew et al., 2009). This indicates that cells with autophagy deficiency lose some advantage and gain some for tumorigenesis. Different oncogenes create different physiological requirements thereby manifesting the autophagy-deficiency associated disadvantage or advantage.

#### **Autophagy sustains mitochondrial energy generation in Ras-expressing cells during starvation**

Ras-expressing cells were more sensitive to glucose or glutamine deprivation in comparison with the vector control cells (data not shown). In addition, Ras-expressing cells continued to proliferate even under nutrient and growth factor deprivation (Figure



3.1D). Ras-expression deferred the downregulation of the energy-consuming mTOR pathway during starvation as indicated by sustained phospho-S6 (data not shown). Therefore, Ras-expressing cells have increased metabolic demand and are less able to reduce metabolic expenditure during periods of starvation.

Under starvation, autophagy deficiency caused energy depletion associated with massive apoptosis in Ras-expressing cells (Figures 3.6B and 3.1C). Autophagy may provide a mean for Ras-expressing cells to use internal resource to meet the bioenergetic demand and survive when nutrients are limiting. During periods of starvation, it is critical for cells to utilize nutrients efficiently. Mitochondrial oxidation phosphorylation is a more effective way than glycolysis to generate energy (Pfeiffer et al., 2001). In autophagy-deficient Ras-expressing tumors, there was accumulation of abnormal swollen mitochondria (Figure 3.5A). This arises a possibility that defective mitochondrial autophagy causes accumulation of abnormal mitochondria and reduced mitochondrial functionality, thereby leading to an energy crisis during starvation. In supporting this, disruption of mitochondrial oxidation phosphorylation with the uncoupler CCCP caused loss of viability under starvation (Figure 3.6A).

In addition to energy generation by oxidation phosphorylation, mitochondria also contribute to biosynthesis by providing TCA cycle intermediates for *de novo* synthesis of fatty acids and non-essential amino acids (DeBerardinis et al., 2008). Whether this function of mitochondria, which is also preserved by mitophagy, is essential for supporting viability of Ras-expressing cells under starvation remains unclear.

### **Mitochondrial phenotype in autophagy-deficient Ras-expressing cells**

In autophagy-deficient Ras-expressing cells, mitochondria were easier to lose membrane potential in starvation than autophagy-competent ones (Figure 3.5B), suggesting a reduced mitochondrial function. This may result from insufficient substrate supply for mitochondrial metabolism or/and failure of repair and elimination of damaged mitochondria during periods of starvation. Lack of substrates leads to mitochondrial malfunction and may increase ROS production that causes damage. Reduced oxygen consumption and depletion of TCA cycle metabolites in both normal growth conditions and starvation (Guo et al., 2010) indicate reduced mitochondrial functionality in autophagy-deficient cells.

How failure of elimination of abnormal mitochondria reduces the whole mitochondrial functionality remains unknown. Abnormal mitochondria and the corresponding ROS production may amplify the abnormality. As mitochondria undergo fusion and fission, lots of damaged mitochondria may functionally or genetically poison those that are functional. Additionally, if cells have the mechanism to control mitochondrial mass, these damaged mitochondria may prevent cells to generate new ones. This is especially detrimental during starvation when cells division, which can dilute the damaged mitochondria, is slowed down.

### **Abnormal mitochondria in autophagy-deficient Ras-expressing tumors**

Autophagy-deficient Ras-expressing allograft tumors in mice showed accumulation of abnormal swollen mitochondria. Whether this is the cause that impaired tumorigenesis or is an effect associated with autophagy deficiency needs further investigation. *in vitro* CCCP addition caused loss of viability under starvation regardless of autophagy competency, indicating that dissipation of mitochondrial membrane potential is

detrimental during period of nutrient/growth factor depletion. Of note, the toxicity of CCCP may not be specific to mitochondrial function of oxidation phosphorylation. Examining whether supply autophagy-deficient Ras-expressing cells with normal mitochondria can rescue the phenotype of sensitivity to starvation will help to clarify this concern.

### **Autophagy deficiency does not complete abolish the growth of Ras-expressing tumors**

Autophagy deficiency impaired tumorigenesis of Ras-expressing cells, but did not complete abolish the tumor growth. This may result from intrinsic variation of phenotype of autophagy deficiency such as mitochondrial functionality and growth of cells that get adequate nutrient supply or upregulate glycolysis to compensate mitochondrial function. Since the growth of tumors did not show an apparent latent stage, this is probably not due to gain of a second mutation in the genome. However, it is possible that tumors overcame the growth disadvantage by acquiring mitochondrial mutations, which occur more frequently than genome mutations do.

### **Exploiting the autophagy dependency in cancer cells with Ras activation for therapeutic purposes**

Autophagy dependency of Ras-expressing iBMK cells is revealed when cells are subjected to starvation, indicating that in Ras-expressing cells, autophagy plays a role when nutrients are limiting. This is consistent with the phenotype of autophagy-deficient Ras-expressing allografts, smaller in tumor sizes and less able to maintain cellular health in the middle of tumors. The findings suggest that autophagy is likely required for human cancers that have aberrant Ras activation to survive and grow in tumor microenvironment,

where nutrients are commonly limiting. In supporting this, administration of CQ, a lysosomotropic agent that inhibits autophagy prolonged the animal survival in a Kras-driven genetic mouse model of pancreatic duct adenocarcinoma (Yang et al., 2010b). Moreover, the efficacy of autophagy inhibitors may be enhanced in combination with treatment that limits extracellular nutrient supply or utilization, or more specific with agents that create dependency on mitochondria such as 2-Deoxyglucose, which interferes glycolysis. Since Ras-expressing cells are less able to reduce the energy expenditure (see above), this type of cancer cells is likely more sensitive to the treatment than normal ones.

### **Scope of autophagy dependency**

Ras exerts its effects through several downstream pathways including Raf, PI3K-Akt and Rel-GDS. Whether the autophagy dependency of Ras-expressing cells majorly results from a single downstream pathway or is a compound effect of multiple pathways remains to be investigated. It is also unknown whether this paradigm is applicable to activation of other oncogenes that show increased metabolic demand such as Myc. Examination of autophagy dependency of these oncogenes will help us to understand which character(s) of oncogenes lead to the autophagy dependency and which aspect of role of autophagy is essential for tumorigenesis.

### **Role of p62 in tumorigenicity of Ras-expressing cells**

Deficiency in p62 impaired tumorigenesis of Ras-expressing iBMK cells. This indicates that tumor cells with Ras-expressing need p62 to support viability and proliferate *in vivo*. Since p62 facilitates autophagy substrate delivery to autophagosomes, it is possible that the impairment of tumor formation resulted from autophagy deficiency

due to a lack of p62. Given that autophagy also control the turnover of the multifunctional p62, it is difficult to dissociate the autophagy adaptor function of p62 from other p62 functions. Deletion of the autophagy machinery-interacting domain of p62 is anticipated causing p62 upregulation and enhancing the p62-mediated signaling or protein sequestration. Deletion of the putative substrate recognition ubiquitin-association domain may be another option. However, this may also interfere other p62 functions. The functional redundant protein NBR1 makes data even more difficult to interpret. In this regard, addressing the question that p62 deficiency impairs Ras-mediated tumor formation through impairing autophagy by a loss of function approach, which interferes with other functions of p62, is unfeasible. However, this approach reveals the significance of other functions of p62 in tumorigenesis.

### **Mechanism of CQ- and CCI-779-induced cell death**

*CQ is a lysosomotropic agent that induces lysosomal stress and inhibits autophagy*

CQ is a lysosomotropic agent that induces lysosomal stress. By interfering with lysosomal function, CQ impairs the degradation of autophagic substrates in autolysosomes. CQ has been shown to increase the number and size of lysosomes of treated cells (Maclean et al., 2008). CQ also causes increase of permeability of lysosomal membrane and release of the neutral protease cathepsin D from lysosomes to cytosol, where it contributes to apoptosis and necrotic cell death (Carew et al., 2007; Yamashima and Oikawa, 2009). Thus, while CQ has been extensively used as an autophagy inhibitor, it also provides cell death signal by induction of lysosomal stress.

*CQ in combination with mTOR inhibition promotes cell death by inhibiting autophagy*

In iBMK cells under ischemic (glucose-free and low oxygen) conditions, CQ was less toxic and less efficient in reverse the mTOR inhibition provided protection in autophagy-deficient cells under ischemia than in those autophagy-competent (Figure 4.5E), suggesting that CQ promoted loss of viability partly through inhibition of autophagy.

Under normal culture conditions, most of the observed toxicity of CQ as a single agent in iBMK cells is not from its activity in blockage of autophagic degradation, since autophagy-competent and -deficient cells did not show apparent differences in response to CQ (data not shown). From the energy perspective, cells can utilize extracellular nutrients and did not rely on autophagy to sustain metabolism in this setting. In consistent with this, supplying MP for mitochondrial energy production did not reverse the CQ-caused cell death (Figures 4.7A and B). In contrast, MP substantially reversed the combination of CQ with mTOR inhibition caused cell death in iBMK cells and human RCC4 cells (Figures 4.7A-D), suggesting that CQ potentiates mTOR inhibition-caused cell death mostly through blocking autophagy to provide mitochondrial substrates.

This suggests that mTOR inhibition not only induced autophagy but also created an autophagy dependency of cells. mTOR inhibition, which mimics starvation, may reduce the uptake of extracellular nutrients and glycolysis. Starvation may also shift the metabolism from glycolysis toward mitochondrial oxidation phosphorylation (Vander Heiden et al., 2009), creating a dependence on mitochondrial respiration and elevating ROS production. Indeed, CCI-779 caused the increased ROS levels in mitochondria (Figure 4.7E). In this situation, mTOR inhibition may also cause cells more dependent on autophagic removal of damaged mitochondria to maintain mitochondrial function and

avoid ROS elevation. Concomitant inhibition of autophagy and mTOR will diminish mitochondrial energy generation and cause metabolic catastrophe, leading to extensive ROS production and cell death (Figure 4.7G). If energy crisis is the cause of ROS production thereby subsequently inducing cell death, supplying cells with MP will reduce ROS production and cell death.

*CQ causes lysosomal membrane permeabilization and promotes necroptosis with CCI-779*

CQ and CCI-779 combination induced a hallmark of apoptosis, caspase-3 activation in apoptosis-competent iBMK cells (Figure 4.1), suggesting activation of apoptotic cell death. This treatment also caused cell death in isogenic apoptosis-defective Bax and Bak double deficient cells (Figure 4.2), indicating that it can activate cell death independent of an intact apoptotic pathway. Accumulating evidence indicates that apoptotic stimuli such as tumor necrosis factor (TNF) can trigger a programmed form of necrosis, necroptosis. This process is dependent on the kinase activity of RIP1 and RIP3 (Vandenabeele et al., 2010). In apoptosis-defective cells, the CQ and CCI-779 combination caused cell death can be rescued by the RIP-1 kinase inhibitor necrostatin-1 (Figures 4.3A and B), indicating that CQ in combination with mTOR induces necroptosis. How necroptosis is triggered is not known. It is possible that CQ in combination with CCI-779 causes stress, promotes TNF production of cells and triggers necroptosis. The mechanism how cells execute necroptosis is still unclear; however, evidence suggests that the TNF-initiated cell death signaling events converge at ROS production and lysosomal membrane permeabilization, which contribute to execution of necroptosis (Vandenabeele et al., 2010). Interestingly, CCI-779 caused elevated ROS levels (Figures 4.7E and F) and CQ

can lead to lysosomal membrane permeabilization (see above). Thus, an alternative model that CQ and CCI-779 cause cell death is: CQ in combination with CCI-779 activates the downstream cell death effectors, permeabilization of lysosomal membrane and elevation of ROS concurrently, and causes necroptosis.

### **Specificity of modulation of autophagy**

Modulation of autophagy inevitably has broad effects, since autophagy is involved in diverse facets in physiology. However, this also indicates that modulation of autophagy encompasses various physiological aspects. For disease prevention, considering the case of aging (cancer initiation and other disorders in a broad definition also) is a kind of functional degeneration, modulation of autophagy—reinstallation of an evolutionarily conserved default cellular function—is probably a way in rejuvenation. In the case of cancer therapy, the focus of autophagy modulation will be exploiting the intrinsic or the therapeutics-induced autophagy dependencies to selectively eliminate the malignant cells.

### **Future perspectives**

Our knowledge of role of autophagy in health and diseases is still incomplete. Further investigation is necessary before we will be able to modulate autophagy for preventive or therapeutic purposes. In disease prevention, the key challenge will be identifying in which aspects—cell refreshment, metabolic regulation and/or adaptation to stress autophagy—is crucial in maintaining well-being of cells in a specific tissue. In disease therapy particularly in cancer treatment, the altered physiology of cells with cancer mutations that creates autophagy dependency can be exploited as a specific therapeutic target. The major challenge of cancer therapy will be identifying what kind of cancer cells (cell types, genetic mutations and cancer stages) rely on autophagy for survival or



more specifically in which way autophagy supports their survival. In addition, since autophagy is a cytoprotective pathway, its induction by cancer therapy may be therapeutically counterproductive. Combination autophagy inhibition with conventional cancer therapies may be a strategy to improve the therapeutic efficacy (White and Dipaola, 2009). To fulfill this, the first step will be examining whether autophagy is induced by a specific therapeutics in a specific type of cancer and whether the treated tumor cells depend on the induced autophagy for survival.

The available pharmacological modulators of autophagy are great tools for us to manipulate autophagy and to explore the relevance of autophagy in different cellular contexts, *in vitro* or *in vivo*. Various tissue specific knockout models of autophagy in mice have increased our understanding of role of autophagy in normal physiology and disease development (Levine and Kroemer, 2008; Mizushima and Levine, 2010). Combination of conditional or inducible autophagy ablation with disease models will help us to better appreciate the interplay between autophagy and diseases.

In addition to the preclinical findings, the information obtained from clinical pathology and epidemiological studies will also inform the role of autophagy in a specific disease and the development of therapeutic regimens for disease prevention and treatment. In this regard, development of probes or markers that detect autophagy occurrence *in vivo* or in disease/tumor samples will help us to better characterize autophagy status in diseases. Genome-wide association study has linked variants of autophagy genes with diseases such as Crohn's disease. By virtue of intensive follow-up studies in disease models, role of autophagy in these diseases has started becoming clear. Given that cancer is a complex process and autophagy exerts its effect in multiple ways, role of autophagy

in tumorigenesis is context-dependent. Further understanding of the role of autophagy in specific contexts will bring autophagy modulation applicable to cancer treatment.

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Bray, K.\*, **Chen, H.-Y.\***, Karp, C.M., May, M., Ganesan, S., Karantza-Wadsworth, V., DiPaola, R.S., White, E. Bcl-2 modulation to activate apoptosis in prostate cancer. (2009) Mol Cancer Res 7, 1487-96. \*authors contributed equally to the work.

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